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THE EFFECT OF INFLAMMATION AND OTHER FACTORS ON THE MOVEMENT OF RADIOACTIVE GLASS PARTICLES FROM THE PERITONEAL CAVITY.

A. D. BANGHAM, P. N. MAGEE AND S. B. OSBORN.

From the Department of Morbid Anatomy, University College Hospital Medical School, and the Physics Department, University College Hospital, London.

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THE work of Menkin (1930, 1931, 1940) on the mechanism of inflammation is well known. The existence of an effective mechanical barrier to the lymphatic drainage of particles and bacteria in the earlier stages of acute inflammation, however, has been questioned (Rich, 1936).

It was felt that the problem could be best investigated by the use of particles similar in size and shape to bacteria, but free from the complicating factors inherent in the living micro-organism.

In previous investigations of the behaviour of particulate matter after injection into animals, various materials have been used (Drinker and Churchill, 1927; Bolton, 1921; Higgins, Beaver and Lemon, 1930; Simer, 1948), but none has permitted easy and accurate quantitative determination in the tissues. The incorporation of a radioactive isotope into the particle makes such a determination possible, and we decided to use glass as a vehicle containing ^{134}caesium. Further, glass particles can be prepared in spherical and amorphous form, and any required size selected.

The present paper describes experiments in which such particles were injected into normal and inflamed peritoneal cavities of rats and mice and the amount reaching the mediastinal lymph nodes measured under varying conditions.

MATERIALS AND METHODS.

Preparation and Properties of Radioactive Material.

The properties of many radioactive materials were investigated before ^{134}Cs was chosen. Its radiation properties are: gamma-ray energy, 0·57, 0·60, 0·79, 1·35 (weak) MeV; beta-ray energy, 0·66, 0·09 MeV; half-life, 1·7 years; excitation cross-section, 25·6 barns; specific activity induced in 4 weeks' irradiation, 5·6 mC./g. This material was selected for the following reasons:

1. It emits β and γ radiations of such energies that end-window β counting and autoradiography are easily possible, while *in vivo* gamma-ray measurements may be made without excessively heavy lead shields.

2. Its long half-life enables a number of experiments to be performed with each batch of material. During the 2 months taken over the experiments to be described decay amounted to less than 7 per cent, so that the activity could be regarded as constant.

3. It is an irradiation product of ordinary caesium, and has a large enough capture cross-section for pile neutrons to enable us to obtain substantial activity in a small amount of material.

4. Caesium can be incorporated in glass quite easily. Messrs. Chance Bros., Ltd., kindly prepared such a glass for us, to the following composition: SiO_2 75·7, CaO 7·5, Na_2O 8·0, Cs_2O 8·0, Al_2O_3 0·9.

The amorphous particles and glass microspheres were prepared from the inactive glass, and then irradiated in the nuclear reactor at Harwell. A second "control" batch of amorphous particles was made from a glass identical except that its Cs_2O was replaced by Na_2O, and this was irradiated beside the first batch. Radioactivity is induced in the Na, Si and Ca as well as in the Cs. After withdrawal from the pile both batches were stored for a week, at the end of which the control batch showed quite negligible radioactivity when compared with the main batch, indicating that the radioactive material used in these experiments was almost entirely ^{134}Cs. Clearly, in very finely divided form, even glass may be slightly soluble in water, and this could invalidate some of our results. This was tested by dialysing a saline suspension of radioactive glass particles, but after 24 hr. not more than 5 per cent of the radioactivity of these particles was found in the dialysate.

Lymph nodes, or other tissues, containing small quantities of glass to be assayed were mounted on nickel planchettes (Putman and Boxall, 1947) in a lead castle near to an end-window Geiger counter (G.E.C. type EHM2). Ideally, the specimen should be reduced to a negligible thickness, to ensure that any beta-ray self-absorption is negligible. However, the beta-rays from ^{134}Cs have a fairly high energy (up to 0·7 MeV). Ashing was found not to be satisfactory, as the following experiment shows: lymph node placed wet in planchette, 524 counts/min.; lymph node dried to constant weight, 830 counts/min.; lymph node ashed at 600° for 48 hr., 66 counts/min.

Clearly, some of the radioactive material is lost from the planchette during the ashing process. As a result, all beta-ray assays were performed by drying the specimen until its counting rate (and therefore its mass) was constant. This technique is admittedly not ideal, as it involves an inaccuracy of a few per cent, which depends on the actual amount of tissue on the planchette. However, such inaccuracy could be tolerated without invalidating any of our experimental findings, and the method was much simpler and quicker than any more accurate method. The sensitivity of the method chosen is shown by the fact that 100 counts/min. (which can be estimated with an accuracy of ± 13 per cent over one minute's count) corresponds to 0·06 microgram of the glass.

In vivo assay of radioactivity was achieved by using a gamma-ray Geiger counter (Twentieth Century type G4) in a massive lead shield. The Geiger counter cannot receive radiation except from an area 1·7 cm. in diameter, and any skin mark can be accurately aligned in the centre of this area. This method of counting is much less sensitive than the former, 100 counts/min. corresponding to about 185 μg. of the glass.

EXPLANATION OF PLATES.

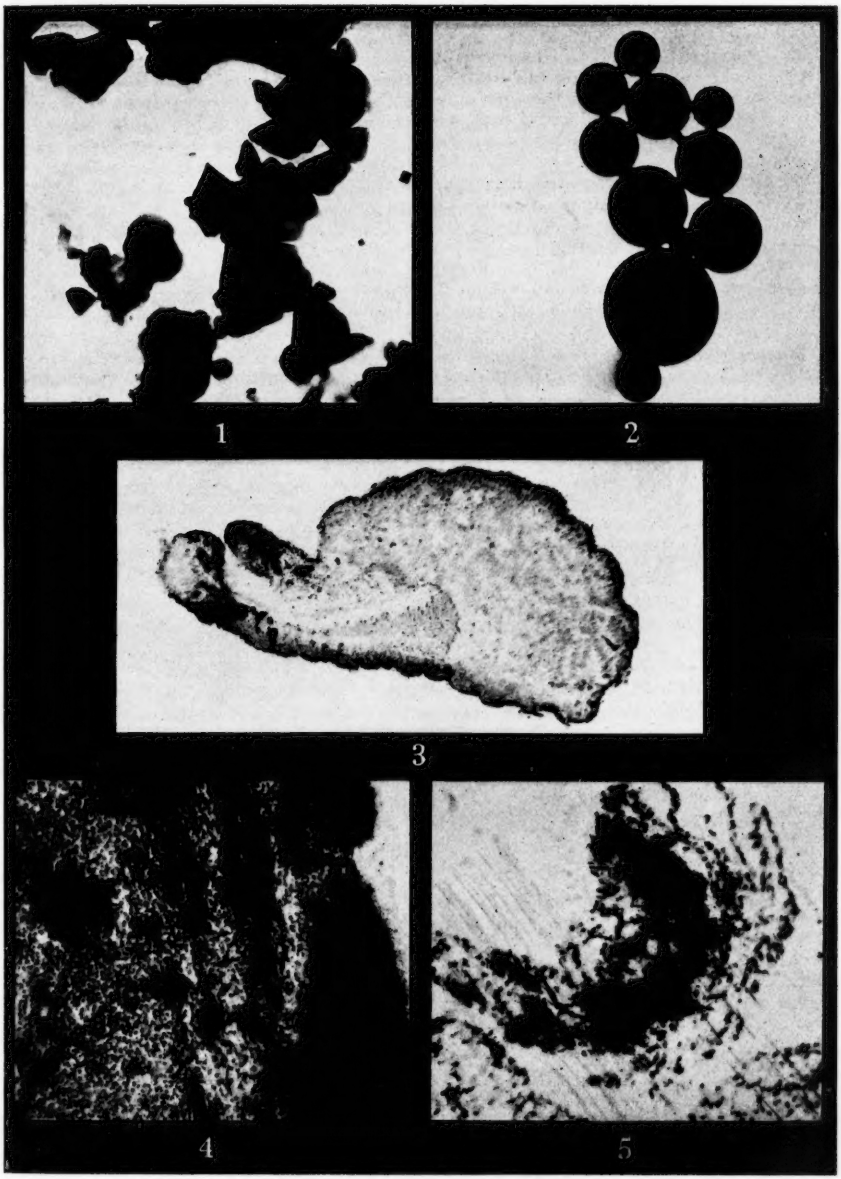
FIG. 1.—Electron photomicrograph of amorphous particles (approx. × 4000).

FIG. 2.—Electron photomicrograph of microspheres (approx. × 6000).

FIG. 3.—Autoradiographic preparation of mediastinal lymph node to show distribution of amorphous glass particles (× 25). Exposure of 72 hr. Ilford G 5 stripping emulsion.

FIG. 4.—Autoradiograph, counterstained with haematoxylin, of omentum 36 hr. after injection of amorphous glass particles, showing inflammatory reaction with cellular infiltration and concentration of particles (× 75).

FIG. 5.—Similar preparation to Fig. 4, 36 hr. after injection of microspheres, showing minimal reaction (× 75).



Bangham, Magee and Osborn.

The raw material, in the form of small pieces of glass, was ground dry in a large mechanical pestle and mortar to a consistency of face powder, and was then dried overnight in an oven at 100°. Using the method of Sollner (1939), the powder was blown through a coal gas flame in a current of oxygen, and the rounded particles were collected in water.

The suspension of microspheres so formed was filtered three times through paper (Whatman 54), and the residue washed with a large volume of distilled water. The combined filtrates containing particles of less than 3–5μ were then centrifuged at 1500 **g.** for 15 min. From time to time an apparent gel formation was observed in the supernatant at this stage, and suggested the presence of colloidal glass.

The deposits were pooled, and subjected to repeated centrifugation at two different speeds. Using water as the suspending medium arbitrary **g** values of 19·0 and 1200 for 3 min. were found to yield a fairly uniform population varying in size from 0·5 μ to 1·5 μ.

The amorphous powder was treated in the same way after grinding, and was found to yield a population of similar size range.

The graded particles were finally dried and placed in standard weighed aluminium containers for dispatch to the neutron pile. Electron photomicrographs are shown in Fig. 1 and 2.

Suspensions of the tracer were prepared by wetting weighed quantities with absolute alcohol, and adding a known volume of 0·9 per cent NaCl. We found it convenient to add a small quantity (up to 100 mg. per cent) of trypan blue in order to verify that the injections went into the peritoneal cavity.

Successful intraperitoneal injections were most frequently achieved with the animals under light ether anaesthesia; they were then easily put into light metal containers which could be placed in any required position.

The mediastinal lymph nodes were dissected out individually from rats and mice and transferred to planchettes for drying and counting. The abdominal cavities were opened to confirm true intraperitoneal injection, and to obtain material for histology when required.

RESULTS.

1. *Distribution of amorphous particles from the peritoneal cavity 1 hour after injection.*

Three rats were given 2·0 mg. amorphous particles suspended in 1·0 ml. intraperitoneally 1 hr. before death. Representative lymph nodes from the cervical, mediastinal, coeliac, para-aortic and superficial inguinal groups were dissected out and assayed for radioactivity.

Whereas the mediastinal group of glands was found to contain 20 μg., no other group contained more than 0·06 μg. These results confirm that particles drain from the peritoneum almost entirely to the mediastinal group of glands. Equivalent weights of liver, spleen and thymus were also examined and found to contain almost no particles.

Similar results were obtained using suspensions of microspheres. The distribution of particles in a single mediastinal lymph gland is shown in an autoradiograph in Fig. 3.

2. *The effect of position of the animal on the clearance of amorphous particles from the peritoneal cavity of rats and mice.*

Forty-nine rats weighing 100–200 g. were divided into 3 groups. All received 200 μg. of amorphous particles intraperitoneally 1 hr. before death. After injection the animals of group A (Fig. 6) were kept in the containers with their heads down, *i.e.*, at an angle of 45°, while those of group B were maintained with their heads up. The remaining rats were allowed to move freely in their cage.

Applying Fisher's "t" test to the means of the logarithmic values obtained in groups A and B, the difference between the means was found to be significant $P < 0{\cdot}01$), which suggests that fewer particles are removed from the peritoneal

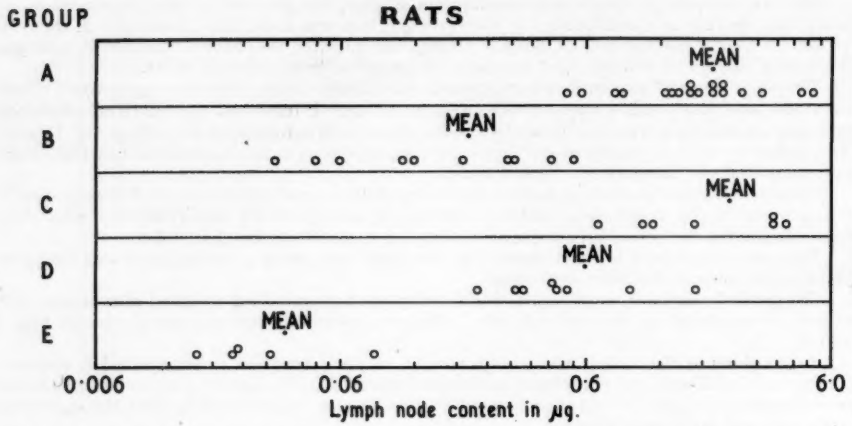


FIG. 6.—Particle content of mediastinal lymph nodes 1 hr. after intraperitoneal injection of 200 μg. (Exp. 2, 7 and 8).

A. Control rats, head down.
B. Rats, head up.
C. Turpentine inflammation, 1 hr.
D. Turpentine inflammation, 24 hr.
E. Severe haemorrhagic inflammation, 1 hr.

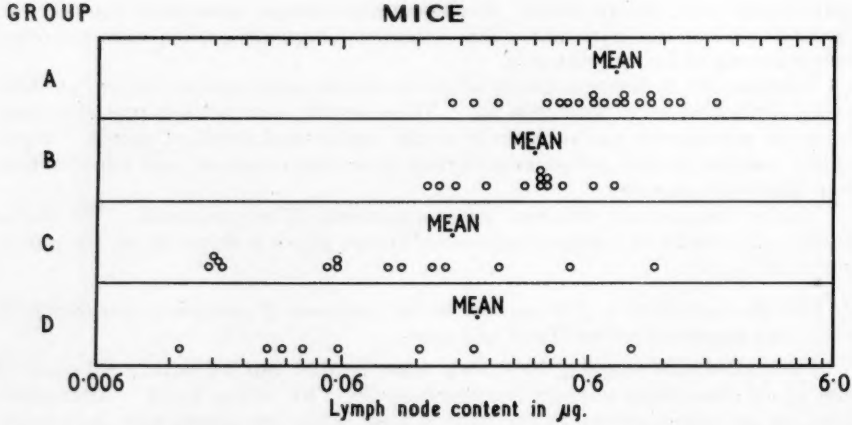


FIG. 7.—Particle content of mediastinal lymph nodes 1 hr. after intraperitoneal injection of 100 μg. (Exp. 2 and 7).

A. Control mice, head down.
B. Mice, head up.
C. Turpentine inflammation, 1 hr.
D. Turpentine inflammation, 24 hr.

cavity when animals are maintained with their heads up. Those allowed to move about freely gave widely varying results intermediate between groups A and B. It was decided, therefore, to carry out all short term experiments with animals in the head down position.

A similar experiment was performed with adult mice except that the dose of tracer was 100 μg., and the same type of result was obtained (Fig. 7).

3. *The effect of varying the weight of particles injected intraperitoneally on their clearance to the mediastinal glands.*

Suspensions were prepared to contain 0·02, 0·2, 2·0 and 20 mg./ml. of amorphous particles.

Twenty young male rats, 100–150 g. body weight, were assembled into 4 groups. Group I received 0·02 mg., group II 0·2 mg., group III 2·0 mg., and

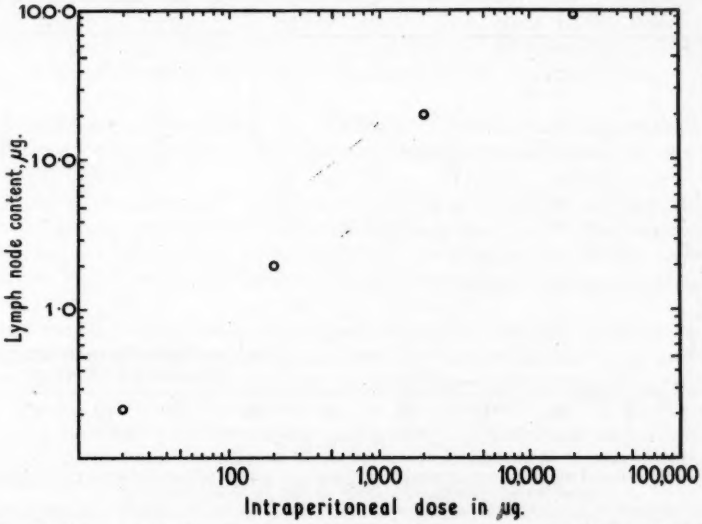


FIG. 8.—Particle content of mediastinal lymph nodes 1 hr. after varying intraperitoneal injections (Exp. 3).

group IV 20 mg. intraperitoneally. The animals were kept head down for 1 hr. before their mediastinal nodes were assayed.

The result of this experiment is shown in Fig. 8. Over this wide dose range the percentage clearance is approximately constant and represents 1 per cent of the intraperitoneal dose. Shortage of radioactive material prevented larger doses from being used.

4. *Comparison of the clearance of amorphous particles and microspheres over varying periods of time.*

A constant weight (200 μg. in 1 ml.) of amorphous particles was injected intraperitoneally into 37 male rats. Groups of animals were killed at varying intervals after injection and their mediastinal lymph nodes assayed for radioactivity. A

similar experiment was done with the same dose, this time of microspheres, in another group of male rats of similar weight. Results are shown in Fig. 9.

Pieces of omentum and parietal peritoneum were removed from all the animals for microscopic examination, and showed that the amorphous particles had induced an acute inflammatory reaction by the end of 24 hr. Autoradiography disclosed collections of particles within the omental inflammatory foci (Fig. 4). Microspheres, in contrast, appeared to provoke only a very slight inflammatory reaction in omentum and peritoneal wall, even after 48 hr. contact (Fig. 5).

5. *Direct measurement of the accumulation of amorphous particles in the mediastinal lymph nodes using a directional γ counter.*

In this experiment two rats weighing approximately 250 g. were given 2·0 mg. amorphous particle suspension intraperitoneally. The rats, kept under light ether anaesthesia, were placed on the lead shield of the counter so that their

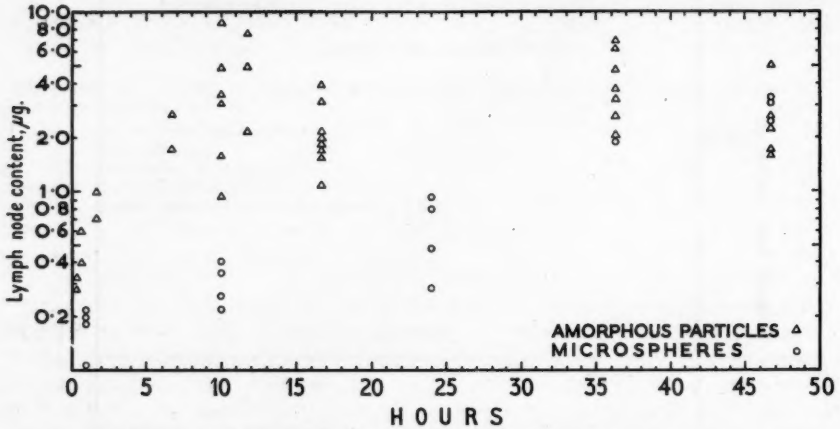


FIG. 9.—Particle and microsphere content of mediastinal lymph nodes at varying time intervals after intraperitoneal injection of 200 μg. doses (Exp. 4).

mediastinal lymph nodes were approximately in line with the window ; the position of the rats was varied slightly, and the highest count obtained over the sternum recorded. These observations were made daily for two weeks. Curves of lymph-node content plotted against time had a similar shape to that obtained in Experiment 4 (see Fig. 9), *i.e.*, a plateau was reached at about 24 hr. and then no increase in lymph-node content for the next 21 days.

On the 21st day the rats were killed and their mediastinal nodes dissected out and assayed on the same counter. The values obtained corresponded well with those observed during life, indicating that the method of counting *in vivo* had been satisfactory.

6. *The clearance of a second injection of amorphous particles from the peritoneal cavities of rats already containing particles.*

Six adult male rats weighing approximately 150 g. were injected intraperitoneally with 200 μg. of amorphous particles. Thirty-six hours later a further

2·0 mg. of suspension was given and the animals were kept with their heads down for 1 hr. before being killed.

A control group of 10 male rats received a single injection of 2·0 mg. of amorphous particles and were killed after being kept for 1 hr. with their heads down. The clearance of particles in the two groups of animals was compared, and application of Fisher's "t" test to the difference between the mean logarithmic values of the two groups reveals a P value between 0·05 and 0·1, but, having regard to the probable content of the nodes due to the first injection alone (*ca.* 3·9 μg., at 1 hr.), the difference is significant.

7. *The effect of a turpentine peritonitis on the clearance of amorphous particles in rats and mice.*

Thirty-three adult male rats were segregated into 3 groups, Group A comprised 18 control animals, group C (7) animals received 0·1 ml. turpentine intraperitoneally 1 hr., and group D (8) animals the same dose 24 hr. before injection of the tracer particles. Each rat was given 200 μg. of amorphous particles and kept in the head-down position for 1 hr. after tracer injection before they were killed.

The results are shown in Fig. 6. Fisher's "t" test shows no significant difference between groups A and C, but there is a significant difference between groups A and D ($P < 0{\cdot}01$).

A similar experiment was performed with 42 adult mice. Group A included 19 control animals, group C (14) animals which received 0·02 ml. turpentine for 1 hr. and group D (9) animals given 0·02 ml. turpentine for 24 hr. The results are shown in Fig. 7 and the difference between the means of all groups is significant ($P < 0{\cdot}01$).

A post-mortem examination was made in every instance in order to confirm the presence of peritonitis and to make sure that the tracer had been injected into the peritoneal cavity. Both rats and mice inflamed with turpentine showed an exudate even 2 hr. after the injection. In the peritonitis of 24-hr. duration the viscera and parietal peritoneum were obviously hyperaemic and fibrinous adhesions were often present. These findings were in contrast to the normal naked-eye appearance of the control animals receiving particles only.

Histologically, peritonitis of 1 hr. duration showed extreme hyperaemia of the omentum with extravascular collection of polymorphs; at 24 hr. the picture was essentially similar, but with fragments of fibrin adherent to the peritoneal surfaces. The control animals showed no microscopical reaction at 1 hr.

8. *Clearance of particles in rats with severe haemorrhagic exudate.*

With the injection of 0·2 ml. turpentine a severe haemorrhagic peritonitis was induced in 5 adult male rats 1 hr. before the injection of the particles. The mean uptake by the mediastinal lymph nodes in these rats amounted to less than 0·06 μg., representing a very considerable reduction in drainage when compared with the expected clearance of 2·0 μg. (Fig. 6).

DISCUSSION.

Two main advantages can be claimed for the use of radioactive glass as a material in the study of particle movement in animals. Firstly, it has been possible to measure quantitatively and with a high degree of specificity the clear-

ance of particles from the peritoneum to the regional lymph nodes, achieved both *in vivo* and by estimation in isolated lymph nodes. Secondly, glass can conveniently be prepared in a variety of sizes and shapes, the glass particles being readily graded into size populations or rounded into microspheres.

Nearly all the earlier methods used for the study of particle movement have depended upon subjective histological or visual observations, or upon arbitrary comparisons of stain intensities, neither method being really capable of accurate measurement. A notable exception was the use of particulate manganese dioxide estimated chemically in the tissues by Drinker and Shaw (1921). Experiments with particles of widely different composition, *e.g.*, yeast cells, milk, red blood cells and various inanimate particles, can only be considered as comparisons between one material and another, and not necessarily as comparisons of size or shape.

One disadvantage of radioactive glass arises from doubts about its inertness, but even this may, within limits, be used to advantage. Amorphous glass particles evoke a fairly acute inflammatory reaction within a few hours ; they may be used, therefore, to study the effect of inflammation on the clearance of particles from the peritoneum. On the other hand, glass microspheres, of about 1–2μ diameter appear to be fairly innocuous, and therefore are preferable as tracer substances. A similar distinction has been noted by Silverman and Moritz (1950).

Another disadvantage comes from the dialysable fraction of the suspensions of glass particles, which is of the order of 5 per cent of the total radioactivity, but if it can be assumed that such dialysable substances would anyhow diffuse freely throughout the animal body, the amount present in a small mass of tissue, such as a lymph node, could be neglected.

Although it is difficult to identify glass particles in stained histological sections, it is comparatively easy to demonstrate such particles of any size by means of autoradiography. This method may, however, be unsuitable for accurate intracellular localization.

The main objection to the use of a radioactive material is, of course, its radioactivity. This is unavoidable, but there is some evidence that in the doses we have used no gross morphological changes characteristic of irradiation were induced. The calculation of radiation dose to the peritoneum is not an easy one. A rigorous calculation must make a number of unverifiable assumptions about the way the glass particles or microspheres distribute themselves either in the space into which they are injected, or on the lining of that space. If we assume (and this assumption leads to the highest value of dosage) that the injected material is quickly deposited uniformly over the peritoneum, and that the area of this is 150 sq. cm., the cells of that lining will be exposed to about 100 r. per day for an injection of 200 μg. Since the total radiation dosage necessary to produce visible skin changes (erythema) is about 300 r. (Mackee and Cipollaro, 1946) it would be surprising if any significant physiological change were induced in the peritoneum after one such day's exposure, but some effect might be expected after a few days.

A rough comparison may perhaps be obtained from the work done on the treatment of peritoneal effusions by colloidal radio-gold. Here, 100 mc. of ^{198}Au may be given, and the dose is about 14,000 r. (Walton and Sinclair, 1952). Allowing for the different conditions, we can deduce from this figure that if the glass particles and microspheres are deposited in the same way as the 50–100 mμ.

colloidal gold particles, 200 μg. of our glass particles or microspheres in the rat would give a dose of about 45 r. per day. This figure is in surprisingly close agreement with the figure of 100 r. per day obtained above, bearing in mind the quite different method of calculation. Drinker and Yoffey (1941) summarise the claims that lymphoid tissue is particularly susceptible to irradiation and the first to show evidence of overdosage. We saw no characteristic change in lymph nodes that had stored particles for 20 days.

Florey (1927) emphasises the importance of using particles that do not flocculate in the presence of serum. If amorphous glass particles were to flocculate like indian ink or carmine, one would expect this to be more pronounced with suspensions of greater concentration—that is, a smaller proportion of particles would be free to pass up the lymphatics. In Exp. 3, however, suspensions were used which varied in concentration by a factor of 1000, and the clearance varied by approximately the same factor. From this, we deduce that the same proportion of particles was available for clearance, and that flocculation was unlikely to have occurred to any great extent. Microscopic examination of omentum and of suspensions of glass particles in plasma confirmed this.

Our results confirm the findings of Muscatello (1895) on the importance of posture. We were misled in our earlier experiments by failing to realize that rats with inflamed peritoneums assumed bizarre attitudes when left together in their cages, and this interfered with absorption.

The clearance curve of amorphous particles from the peritoneum to the regional lymph nodes when studied over a period of time requires comment. Our results indicate that although the amorphous particles rapidly accumulate during the first few hours, the total content of the lymph nodes does not obviously increase during subsequent days. That this is probably not due to " saturation " of the glands is shown in Exp. 3, where it appeared that lymph nodes could readily accommodate up to 100 times that amount found in the animals. Furthermore, it cannot be explained by clearance elsewhere or by loss from the lymph nodes themselves, since replenishment of the tracer reservoir with a massive intraperitoneal injection fails to increase the lymph-node content significantly.

Microspheres, on the other hand, are cleared more slowly, and the amount in the lymph nodes was still increasing after 48 hr. Histological evidence clearly suggests that when amorphous particles are injected intraperitoneally they evoke an acute inflammatory reaction, capable not only of fixing the particles *in situ*, but of preventing further particles from reaching the regional lymph nodes. But Fig. 9 shows that the amorphous particles are cleared more rapidly during the initial hours after injection and long before fixation in inflammatory zones is apparent.

Furthermore, amorphous particles are removed very much faster than the less irritant microspheres, suggesting that in the earlier stages of inflammation lymphatic drainage of particles is enhanced rather than obstructed. This is consistent with the findings of Miles (personal communication, 1952), who was able to show the existence of patent lymph channels within early severe inflammatory foci by injection of indian ink.

Although our findings afford some support for the contention of Menkin (1940), who believes that fixation of particles occurs in and around inflamed areas, we believe that this mechanism is operative only in the fully developed inflammatory lesion. If one may draw a comparison between glass particles and bacteria, the

fixation of bacteria at the site of inoculation would be more likely to be due to an immune response than to any mechanical obstruction.

By measuring the clearance of particles from peritoneal cavities in which inflammation of varying intensity and maturity had been induced, we have shown that in rats, 1 hr. after the injection of a small dose of turpentine a peritonitis was present, but no reduction in clearance of particles was found. After the same dose had been in contact with the peritoneum for 24 hr., however, there was a significant fall. In severe haemorrhagic peritonitis produced by massive injections of turpentine, there was almost no movement of particles from peritoneum to lymph gland. These animals, however, were comatose and severely shocked, so that factors other than local inflammatory processes were present.

These observations show that both severity and duration of inflammation determine the rate at which particles leave the inflamed peritoneum.

SUMMARY.

The preparation and properties of minute amorphous and spherical radioactive glass particles containing ^{134}caesium are described, and a quantitative account of their behaviour after intraperitoneal injection in rats and mice is presented. This method is claimed to have substantial advantages over those used previously.

No peritoneal reaction was observed 1 hour after injection of either amorphous particles or microspheres, but after longer periods of time the amorphous particles provoked an acute inflammatory response.

A comparison of the rates of drainage of the two types of particle from the peritoneal cavity to the mediastinal lymph nodes suggested that the inflammatory response provoked by the amorphous particles increased their speed of removal *via* the lymphatics initially, and only later caused their fixation at the site of inflammation.

The effect of peritonitis due to turpentine was studied, and it was concluded that there is little or no mechanical obstruction to the lymphatic drainage of particles during the earlier stages of such an inflammation.

Previous observations concerning the route and the effect of posture on the clearance of particles from the peritoneum were confirmed.

We should like to express our thanks to Professor G. R. Cameron, F.R.S. for his advice and encouragement. We are indebted to Dr. Bastick of Chance Bros., Ltd., for advice and the preparation of the special glass used ; to Dr. Challice of the National Institute for Medical Research, for electron micrographs, to Dr. Please for assistance in the statistical analyses, and to Mr. V. K. Asta for the diagrams.

REFERENCES.

Bolton, C.—(1921) *J. Path. Bact.*, **24**, 429.
Drinker, C. K., and Churchill, E. D.—(1927) *Proc. roy. Soc.*, B, **101**, 462.
Idem and Shaw, L.—(1921) *J. exp. Med.*, **33**, 77.
Idem and Yoffey, J. M.—(1941) ' Lymphatics, Lymph and Lymphoid Tissue.' Harvard (University Press), 1st ed.
Florey, H. W.—(1927) *Brit. J. exp. Path.*, **8**, 479.
Higgins, G. M., Beaver, M. G., and Lemon, W. S.—(1930) *Amer. J. Anat.*, **45**, 137.

MACKEE, G. M., AND CIPOLLARO, A. C.—(1946) ' X-rays and Radium in the Treatment of the Skin.' London (Kimpton).
MENKIN, V.—(1930) *J. exp. Med.*, **51**. 879.—(1931) *Ibid.*, **53**, 647.—(1940) ' Dynamics of Inflammation.' New York (Macmillan.).
MUSCATELLO, G.—(1895) *Virchows Arch.*, **172**, 327.
PUTMAN, J. L., AND BOXALL, R. H.—(1947) *Brit. J. Radiol.*, **20**, 190.
RICH, A. R.—(1936) *Arch. Path.*, **22**, 228.
SILVERMAN, L., AND MORITZ, A. R.—(1950) *Arch. industr. Hyg.*, **1**, 499.
SIMER, P. H.—(1948) *Anat. Rec.*, **101**, 333.
SOLLNER, K.—(1939) *Industr. Engng. Chem.* (*Anal.*), **11**, 48.
WALTON, R. S., AND SINCLAIR, W. K.—(1952) *Brit. med. Bull.*, **8**, 165.

SOME FACTORS INFLUENCING THE RESPONSE OF ANIMALS TO IMMUNISATION WITH COMBINED PROPHYLACTICS.

MOLLIE BARR AND MONA LLEWELLYN-JONES.

From the Wellcome Research Laboratories (Biological Division), Beckenham, Kent.

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IT has long been known that " crowding-out " of antibody formation may occur when an antigen is injected into an animal at a time when active production of another antibody is occurring. Benjamin and Witzinger (1912) found that a preliminary injection of horse serum inhibited the formation of haemolysin to sheep cells. Lewis (1915) found that an amount of protein that produced a marked anaphylactic sensitisation in a guinea-pig when it was injected alone failed to do so when it was injected together with or 24 hours after a larger dose of another protein. He expressed the view that the number of receptors in the body that can unite with a foreign protein is limited, and that the inhibiting protein, if present in large amounts, combines with most or all of these receptors.

Glenny, Hopkins and Waddington (1925) found that an injection of diphtheria toxin-antitoxin mixture or of toxoid into diphtheria-immune animals evoked little or no response if it were given at a time when the animals were producing precipitins to horse, cow or goat serum. In addition active precipitin formation interfered with the primary response of guinea-pigs to diphtheria toxoid. Glenny and Waddington (1928) found that interference with the response to an injection of diphtheria toxoid occurred in guinea-pigs injected at the same time or 2, 4 or 6 days after an injection of normal horse serum. The interference in all these examples was attributed to the crowding-out of the response by the formation of precipitins in response to an injection of heterologous protein. In addition Glenny and Waddington (1926) showed that in animals injected with a combined diphtheria-scarlet fever prophylactic an excess of scarlet fever toxin interfered with the antigenic action of the diphtheria prophylactic. Thus an excessive antigenic action of one component of a preparation can suppress the active immunity normally evoked by another component, and an injection of antigen given at a time of vigorous production of antibody to another may have little effect. Scheibel (1944) made a careful study of the responses of guinea-pigs to two doses of a combined prophylactic containing, per ml., 50 Lf of diphtheria toxoid and 25 Lf ($\equiv$ 12·5 International units 1950) of tetanus toxoid adsorbed on aluminium hydroxide. She found the responses to both components of this preparation to be somewhat lower than those to each when injected alone. She also compared the responses to a single injection of different doses of this preparation with those to the same doses of the components when injected alone. The tetanus antitoxin responses in animals injected with the combined prophylactic were always lower, and with the smallest dose used, significantly lower, than those in the controls.

A number of workers have reported an increased response to one antigen when this was mixed with another before injection. We are of the opinion that many of these claims were based on little evidence. For example Lapin (1942) showed a table comparing the tetanus antitoxin titres of *babies immunised by him* (using alternate injections of tetanus A.P.T. plus pertussis antigens and " clear " diphtheria toxoid plus pertussis antigens) with the titres of *men of the United States Navy immunised by Hall* (1940) with tetanus A.P.T. alone. No mention was made of the potency of these prophylactics. The doses injected were " 0·5 ml.," and the " average antitoxic levels " for the two groups were stated to be 0·50 and 0·16 unit/ml. (reference to Hall's original paper shows that his figure was 0·062, not 0·16 unit/ml.). Lapin states in the summary of his results on combined prophylaxis : " tetanus immunity resulted in an increase in the average antitoxic level to 0·50 unit." This statement appears to have been based solely on the comparison between the titres of babies with those of adult sailors, and indeed Lapin states that these groups were " strictly comparable " because two doses of tetanus A.P.T. had been injected at an interval of 8 weeks and tests made 3 months later. Incidentally it is difficult to reconcile Lapin's statement that the titres of his own group were measured 3 months after the second injection of tetanus A.P.T. with the immunisation scheme shown in Table I of his paper. According to the procedure laid down there, three injections of tetanus A.P.T. would have been given by this time : Hall's had definitely had only two injections when the antitoxin titres were measured. But even assuming that the Lf dosage was the same in both groups, the babies would have received about ten times as much antigen, in terms of body weight, as the adults. This work is frequently quoted by other writers as an example of a synergic effect in tetanus immunisation, caused by the use of a combined prophylactic.

Ramon (1936), summarising results obtained by him and his colleagues, gives figures which are supposed to illustrate the beneficial effect on tetanus immunity of using tetanus toxoid plus T.A.B. vaccine for immunisation. Three persons were injected with a combined prophylactic and two controls with tetanus toxoid alone. Neither of the controls produced so much as 0·01 unit/ml. tetanus antitoxin (the titres were 0·0033 and 0·005 unit), and the persons injected with toxoid plus T.A.B. vaccine had titres of 0·033, 0·02 and 0·02 unit/ml. Considering the small number of persons, the low degree of immunity produced and the unsound procedures used by Ramon (Ramon and Richou, 1950) in the determination of low antitoxin titres, we think it regrettable that this work often appears to be regarded as affording a classical example of synergy.

It is not to be denied that synergic effects occur in certain circumstances. Maclean and Holt (1940) recorded higher tetanus antitoxin titres in a group of adults immunised with T.A.B.T. (tetanus toxoid + typhoid-paratyphoid vaccine) than in another group immunised with tetanus toxoid alone. The first dose of the combined prophylactic contained half as much vaccine as the second, but the strength of the tetanus toxoid was the same in both doses. Greenberg and Fleming (1947, 1948), using a single dose method, showed that the Schick-negative rate was significantly higher in guinea-pigs injected with diphtheria toxoid to which had been added pertussis vaccine, tetanus toxoid, both these substances, T.A.B. vaccine, or scarlet fever toxin and pertussis vaccine, than in animals injected with the diphtheria toxoid alone. This increased antigenic effect after addition of tetanus toxoid is in contrast to the findings of Scheibel

(1944), who, however, used these toxoids adsorbed on aluminium hydroxide. The dosage of tetanus toxoid used by Greenberg and Fleming was unfortunately not stated.

Little work has been directed to the study of the antigenic efficiency of combined prophylactics in which the proportions of the components have been varied. Greenberg and Fleming (1947) found that the diphtheria immunity produced in guinea-pigs immunised with combined preparations containing *H. pertussis* vaccine of strength between 20×10^9 and 160×10^9 organisms per ml. was in each case better than that produced by the toxoid alone. From the theoretical standpoint, the antigenic efficiency of combined antigens might be expected to depend on (*a*) the relative proportions of the components, (*b*) the dosage administered, and (*c*) the immunity state of the individuals to be immunised.

It is evident from the literature that some ill-balanced preparations have been used by a number of workers in the immunisation of babies. For example Peterson and Christie (1951), using a combined diphtheria-tetanus-pertussis typhoid preparation, concluded from their results that this material contained insufficient diphtheria toxoid. Thirteen months after the second injection of the course more than a quarter of the children had less circulating diphtheria antitoxin than an amount often considered to be adequate (0·003 unit/ml.). The tetanus antitoxin titres, however, were still satisfactory. The authors suggested that a higher dosage of diphtheria toxoid was necessary, but it appears possible to us that the diphtheria immunity state might have been appreciably improved if the dosage of tetanus toxoid had been reduced, without necessarily reducing the tetanus immunity to an unsatisfactory level. The prophylactic contained per ml. 30 Lf of diphtheria toxoid and tetanus toxoid, corresponding to " 100,000 minimal lethal doses " (probably 50-100 Lf). As judged by the amount of the components generally considered necessary to produce satisfactory immunity when injected alone, tetanus toxoid was present in enormous excess. This may have caused considerable interference with the diphtheria response.

When unbalanced prophylactics are used for the immunisation of non-immune persons, a boosting dose of one component will be required before that of another, and differences in the immunity state may affect the responses to the different components in a boosting dose of a combined preparation.

We are engaged in studying some of the factors that might influence the efficiency of immunisation with combined prophylactics. This paper presents some early results showing the effect of pre-existing immunity to one component on the response to another, and the effect of addition of vaccines on the antigenic efficiency of tetanus toxoid.

EXPERIMENTAL.

The Effect of Pre-existing Immunity to One Component.

This experiment was concerned with the effect of immunity to diphtheria toxoid on the response to immunisation with combined prophylactics containing purified diphtheria and tetanus toxoids adsorbed on a mineral carrier. Three such prophylactics were used. All contained, per ml., 60 Lf of diphtheria toxoid, but the tetanus toxoid content of A was 6 Lf, of B 12·5 Lf, and of C 25 Lf/ml.

TABLE I.—*The Distribution of Tetanus Antitoxin Titres of Normal and Diphtheria-Immune Guinea-pigs after Immunisation with Two Doses of Combined Diphtheria-tetanus Prophylactic.*

Prophylactic used.	Guinea-pigs.	No. of guinea-pigs with titres— Over / Under	– 0·01	0·01 0·02	0·02 0·05	0·05 0·10	0·10 0·20	0·20 0·50	0·50 1·0	1·0 2·0	2·0 5·0	5·0 10	10 20	20 50	50 100	Geometric mean of tetanus response.	Comparison of log. geometric means. *t.*	*P.*	Geometric mean of diphtheria response.
A	Normal		–	–	–	–	–	–	1	3	6	2	3	–	–	3·66	–	–	3·19
	Immune		2	1	0	1	2	0	1	6	1	1	–	–	–	—			—
B	Normal		–	–	–	–	–	–	–	–	2	5	5	2	1	11·4	2·752	*ca.* 0·01	2·44
	Immune		–	–	–	–	1	0	6	1	7	2	4	–	–	4·04			—
C	Normal		–	–	–	–	–	–	–	–	1	2	5	2	1	14·6	3·572	<0·01	1·82
	Immune		–	–	–	1	0	0	3	3	2	5	2	–	–	2·51			—

A. 60 Lf diphtheria toxoid + 6 Lf tetanus toxoid.
B. „ „ „ + 12·5 „ „
C. „ „ „ + 25 „ „

Guinea-pigs with active immunity to diphtheria toxoid were selected from animals that had received the second of two injections of diphtheria prophylactic 6 months previously. Groups of normal animals of the same weights were used as controls. Two doses, each of 0·005 ml. of the combined prophylactics, were injected at an interval of 28 days, and the guinea-pigs were bled 10 days later. From animals injected with preparation B, blood samples were also taken at the time of the second injection. The individual sera were titrated for tetanus antitoxin by the method of Glenny and Stevens (1938), and for diphtheria antitoxin by a modification of the Römer and Sames (1909) guinea-pig intracutaneous method (Glenny and Llewellyn-Jones, 1931).

Table I shows the tetanus antitoxin responses of all the guinea-pigs injected with the combined prophylactics. There is clearly a considerable difference between the titres of the two groups injected with preparation A. Two of the 15 animals with pre-existing diphtheria immunity failed to produce any detectable tetanus antitoxin, and the scatter of titres in this group was considerable. All the controls, however, produced at least 0·5 unit/ml., most of them over 2·0 units. The means of the logarithms of the titres of diphtheria-immune animals injected with prophylactics B and C were significantly lower than those of the corresponding normal control animals. Thus, interference with the tetanus response may occur even when diphtheria-immune animals are injected with a combined diphtheria-tetanus prophylactic of high tetanus toxoid content, such as C.

It is of interest to record the tetanus antitoxin titres of the guinea-pigs injected with prophylactic B, at the time of the second dose, 28 days after the first. These are shown in Table II.

TABLE II.—*The Tetanus Antitoxin Titres of Diphtheria-Immune and Normal Guinea-pigs 28 Days after the First of Two Injections of Combined Diphtheria-tetanus Prophylactic B.*

Guinea-pigs.	No. of guinea-pigs with antitoxin titres (units)/ml.)—					
Over	–	0·01	0·02	0·05	0·10	0·20
Under	0·01	0·02	0·05	0·10	0·20	0·50
Diphtheria-immune	5	2	1	0	4	1
Normal	12	2	0	0	0	1

Twelve of the 15 normal animals failed to produce any detectable tetanus antitoxin after one injection of the combined prophylactic. Eight of 13 of the diphtheria-immune guinea-pigs, however, had shown a definite tetanus antitoxic response, 5 having titres of 0·1 unit/ml. or more : there was insufficient serum to complete tests on samples from 2 animals. This result conflicts with that obtained after two injections of combined prophylactic. The immunity states of the groups of animals differed, however, at the time of each injection. In the normal animals the first injection of combined prophylactic was a primary stimulus of two antigens. The basal immunity established by the time the second dose was injected would be of the same degree for both components since these were well balanced. The diphtheria-immune guinea-pigs. on the other hand, had high titres of circulating antitoxin (0·5–1 to 5–10 units/ml.) at the time of the first injection of combined prophylactic. Although this injection brought about a considerable rise in the diphtheria antitoxin titres, it is probable that much of this antigen was taken up by the circulating antitoxin already present, and ren-

dered temporarily inert. If this occurred the prophylactic would act as a mixture containing a large excess of tetanus toxoid injected into animals with a reduced amount of circulating diphtheria antitoxin. At the time of the second injection the mechanism involved in the production of diphtheria immunity may have been in a highly reactive state. Crowding-out of the response to the tetanus antigen in the second dose of combined prophylactic occurred. It is, however, possible that this interference was due, not to the diphtheria toxoid present in the second dose, but to a prolonged effect of this material in the first dose. It would therefore be interesting to know whether a similar result would have been obtained if the second dose of combined prophylactic had been replaced by one of tetanus toxoid alone. It is possible too that a different result might have been obtained if the diphtheria-immune guinea-pigs had had good potential immunity but little or no circulating antitoxin at the beginning of the experiment. These and several other interesting points arising from this work are being investigated. At present no final explanation should be attempted, for many factors may be involved. It is interesting in passing to note the responses of the normal guinea-pigs to injections of the different prophylactics (Table I). There was a marked difference between the geometric mean tetanus antitoxin titres of those injected with A and B, but C produced hardly better immunity than B. The diphtheria responses were progressively lower as the tetanus content of the prophylactic was increased, although the dosage of diphtheria toxoid was the same for each group.

We have obtained another somewhat similar result in experiments on prophylactics containing a toxoid and a vaccine. A group of guinea-pigs received an injection of 0·05 ml. of T.A.B. vaccine containing per ml. 10^9 *S. typhi* and 5×10^8 each of *S. paratyphi* A and B. One month later an injection of 0·05 ml. of T.A.B.T. (tetanus toxoid plus T.A.B. vaccine) containing the same amount of vaccine was given ; this was repeated after 28 days. Control animals received two injections of T.A.B.T. at the same interval without the preliminary injection of vaccine. All these guinea-pigs were bled 10 days after the second injection of the combined prophylactic, and the individual sera were tested for tetanus antitoxin content. The results are shown in Table III. An additional factor

TABEL III.—*The Tetanus Antitoxin Titres of Guinea-pigs after Immunisation with Two Doses of T.A.B.T.*

Group.	Over / Under	– / 0·01	0·01 / 0·02	0·02 / 0·05	0·05 / 0·10	0·10 / 0·20	0·20 / 0·50	0·50 / 1·0	1·0 / 2·0	2·0 / 5·0	5·0 / 10	10 / 20	20 / 50
		No. of guinea-pigs with antitoxin titres (units/ml.)—											
A	. .	–	–	–	–	–	–	–	1	4	2	2	4
B	. .	1	0	0	0	1	1	1	0	7	3	–	–

Group B had received an injection of T.A.B. vaccine alone, 28 days before the first injection of T.A.B.T.

must be considered as a possible cause of the poorer response shown by the animals that had a preliminary injection of vaccine alone. Whereas in the previous experiment no injections of diphtheria prophylactic had been given for at least 6 months before the first dose of combined prophylactic, the animals in this experiment had received primary stimulation with T.A.B. only a month beforehand. It is possible that the primary response to the injection of T.A.B. was still occurring

at the time of the first injection of T.A.B.T. This may have caused interference with the response to the tetanus toxoid in the first dose of T.A.B.T. : the same result might have been obtained if an entirely different vaccine had been injected. Several experiments are now in progress in an attempt to find the cause of the interference with the response to immunisation with tetanus toxoid in this experiment. We think it as well, however, to record these preliminary results at this stage, for whatever the correct explanation, it is evident that there may be danger in using a combined prophylactic for the purpose of simultaneously boosting the titre of one antibody and producing another in primary immunisation. The response to the new antigen may be greatly reduced, even if it is present in slight excess. The results suggest that unbalanced immunity might continue after the injection of boosting doses of combined prophylactics into persons who had received primary courses of immunisation with ill-balanced mixtures containing too little of one component. It may also be unwise to introduce into courses of immunisation with combined prophylactics additional injections of one antigen alone, though it seems reasonable to suppose that the result of such a procedure would depend on the spacing of injections and the dosage.

The Effect of Adding Vaccine on the Antigenic Efficiency of Toxoid.

In another series of experiments we have studied the effect of addition of typhoid-paratyphoid vaccine on the antigenic efficiency of tetanus toxoid. It is well known that qualitative differences may exist among batches of toxins and toxoids (Barr and Glenny, 1949), and we ourselves (unpublished) have occasionally detected them among batches of tetanus toxoids prepared at these laboratories. It is also possible that the addition of a vaccine might not have the same effect on all batches of toxoid. The same batch of tetanus toxoid was therefore used throughout, both as control and in all the preparations of T.A.B.T. The T.A.B. vaccines were added in the form of a concentrated suspension, so that the effective volume dilution of the tetanus toxoid was less than 10 per cent. The unmixed tetanus toxoid T and three preparations of T.A.B.T. were compared. D was of the strength used by Maclean and Holt as T.A.B.T. No. 1, and contained per ml. 5×10^8 *S. typhi* and $2{\cdot}5 \times 10^8$ each of *S. paratyphi* A and B. Preparation E contained the same batch of T.A.B. vaccine, but in twice the concentration (T.A.B.T. No. 2), and F, also T.A.B.T. No. 2, contained a different batch of vaccine. All contained 0·5 per cent phenol.

The antigenic efficiency of these preparations was compared in normal guinea-pigs of comparable weight : three systems of dosage were used. We have found that when guinea-pigs are injected with two doses, each about 0·5 Lf, separated by an interval of 28 days, a high degree of immunity is usually established. When the dosage is 0·2 Lf on each occasion, differences in antigenic efficiency between preparations can sometimes be detected and some animals fail to respond. The animals in this experiment were bled 10 days after the second injection, and the individual sera titrated for tetanus antitoxin.

The antitoxin titres of animals injected with the larger doses (about 0·5 Lf) are shown in Table IV. A very small scatter of titres was shown by those injected with sample D, and there was no significant difference between the means of the logarithms of the titres of these animals, those injected with E (containing the same batch of T.A.B. vaccine but in double strength) and those that received

TABLE IV.—*The Tetanus Antitoxin Titres of Guinea-pigs after Immunisation with Two Doses (each approximately 0·5 Lf) of Tetanus Toxoid or of T.A.B.T.*

Batch.	Type.	No. of guinea-pigs with antitoxin titres (units/ml.)— Over / Under 0·1 / 0·2	0·2 / 0·5	0·5 / 1·0	1·0 / 2·0	2·0 / 5·0	5·0 / 10	10 / 20	20 / 50	Geometric mean.
T	Toxoid	–	–	–	1	3	3	4	3	8·92*
D	T.A.B.T. 1	–	–	–	–	4	3	8	–	8·26
E	T.A.B.T. 2	–	–	–	1	4	2	2	4	8·60†
F	T.A.B.T. 2	1	1	2	2	2	4	2	1	2·86*†

Batch D (T.A.B.T. No. 1) contained per ml. 5×10^8 *S. typhi* and $2{\cdot}5 \times 10^8$ each *S. paratyphi* A and B : batches E and F (T.A.B.T. No. 2) contained double these amounts and the same amount of tetanus toxoid.

$*t = 2{\cdot}357$ $P = 0{\cdot}028$ for T and F
$\dagger t = 2{\cdot}147$ $P = 0{\cdot}043$ for E and F

tetanus toxoid (T) alone. A considerably larger scatter was shown by the guinea-pigs injected with the second batch of T.A.B.T. No. 2 (F in Table IV), and the mean logarithmic titre of this group was significantly lower than that of the group injected with tetanus toxoid alone, or with the other batch of T.A.B.T. No. 2, E.

TABLE V.—*The Tetanus Antitoxin Titres of Guinea-pigs after Immunisation with Two Doses (each 0·2 Lf) of Tetanus Toxoid or of T.A.B.T. (the Same Batches as in Table IV).*

Batch.	Type.	No. of guinea-pigs with antitoxin titres (units/ml.)— Over / Under – / 0·01	0·01 / 0·02	0·02 / 0·05	0·05 / 0·10	0·10 / 0·20	0·20 / 0·50	0·50 / 1·0	1·0 / 2·0	2·0 / 5·0	5·0 / 10	10 / 20	20 / 50
T	Toxoid	3	0	1	0	1	2	0	2	4	1	1	–
D	T.A.B.T. 1	–	–	–	–	–	–	–	1	9	4	–	–
E	T.A.B.T. 2	1	1	0	0	1	0	0	3	5	2	1	1
F	T.A.B.T. 2	1	2	0	1	0	0	1	4	4	2	–	–

Table V shows the tetanus antitoxin titres of guinea-pigs injected with two smaller doses, each of 0·2 Lf. The best responses were those of the guinea-pigs injected with preparation D (T.A.B.T. No. 1) : the scatter was again very small, the geometric mean titre was slightly less than half that of animals injected with larger doses of this material, and the presence of the vaccine improved the response to the tetanus toxoid. The addition of double strength T.A.B. had no effect on the responses to the toxoid at this level of dosage : if anything the animals injected with T.A.B.T. No. 2 had slightly better titres than those injected with toxoid alone, but there is no striking difference between these groups.

Thus from the data in Tables IV and V it is evident that the addition of one particular batch of vaccine (double strength) had no effect on the antigenic efficiency of tetanus toxoid at either level of dosage in guinea-pigs. The addition of a different batch caused a significant reduction in the response when relatively large doses were injected, but had no effect when smaller doses were given. The addition of the first batch of vaccine, but at half strength, had no effect on the antigenic efficiency of the tetanus toxoid when large doses were injected, but a synergic effect when the somewhat inadequate dose was given. This was the only instance in which a synergic effect was observed.

A comparison was also made of the antigenic effect of a single dose of 1 ml. (9 Lf) of three of these preparations into standard weight guinea-pigs : these animals were bled 4, 6 and 8 weeks after injection, and the tetanus antitoxin titres found at 6 weeks are shown in Table VI : the same general comparative distributions were found at 4 and 8 weeks.

TABLE VI.—*The Tetanus Antitoxin Titres of Guinea-pigs 6 Weeks after a Single Injection of 1·0 ml. (about 9 Lf) of Tetanus Toxoid or of T.A.B.T. No. 2.*

			No. of guinea-pigs with tetanus antitoxin titres (units/ml.)—							
Batch.	Type.	Over / Under	–	0·01	0·02	0·05	0·10	0·20	0·5	1·0
			0·01	0·02	0·05	0·10	0·20	0·50	1·0	2·0
T	Toxoid		–	–	1	0	1	8	3	1
E	T.A.B.T. 2		1	0	2	3	5	3	1	–
F	T.A.B.T. 2		7	0	2	2	0	2	–	–

Better immunity was produced by tetanus toxoid alone than by either of the combined prophylactics. Batch F (T.A.B.T. No. 2) again produced the poorest immunity, less than half the animals injected having any detectable antitoxin 6 weeks later. It is not possible to say whether the inferiority of this preparation in respect of the tetanus antitoxin response was due to crowding-out by a strong antigenic effect of the vaccine. There is every reason to suppose that some batches of vaccine may be far more potent antigens than others, but it is also possible that some interaction may occur between the components of combined prophylactics, and that this may vary with the actual batches mixed.

There was a slight difference between the two prophylactics E and F. Batch E was a routine preparation of T.A.B.T. containing freshly prepared vaccines : our experiments were done 3 months after it was made. The batches of vaccine contained in F were not freshly prepared, but had been harvested at times between 4 and 11 months before they were mixed with the tetanus toxoid ; all had been stored as concentrated suspensions. The batch of T.A.B.T. prepared with these vaccines was used within a few days of mixing. It is possible but rather improbable that the difference in the antigenic efficiency of the same tetanus toxoid in these two batches could be due to these differences in age of the vaccines.

In the routine testing of tetanus prophylactics for issue we have frequently found the antitoxin titres of guinea-pigs injected with a single dose of 5 ml. of batches of T.A.B.T. No. 2 to be lower than those of animals injected with 5 ml. of tetanus toxoids alone. It was indeed this finding that led us to do the experiments recorded here. We wished to know the effect of a smaller single dose (1 ml.), and to see whether the immunity produced by two spaced doses of T.A.B.T. No. 2 was also lower than that produced by similar doses of tetanus toxoid alone. It appears from these few results that tetanus toxoid alone is a better antigen than T.A.B.T. No. 2 when single dose methods are used for the comparison. This may be due to a crowding-out of the primary response to tetanus toxoid by the response to a relatively large dose of vaccine which, however, might be sufficient to produce an adverse effect by causing excessive local damage.

DISCUSSION.

It is clearly desirable to use combined prophylactics for active immunisation in order to reduce the number of injections needed to establish an adequate degree of immunity to a number of different diseases. The preliminary results recorded here show that certain factors may strongly influence the results obtained in immunisation with combined prophylactics. It is possible that these antigens may come into general use in this country before their limitations are fully realised.

A suggestion has been made (Conybeare and Logan, 1951) that " it might be reasonable to offer parents active immunisation against tetanus " for their children " when they are being asked to agree to the boosting inoculation against diphtheria at the time of entry to school." It is possible that some medical officers might give two injections of a combined prophylactic in an attempt to carry out such a programme with the minimum number of injections : an unsatisfactory state of tetanus immunity might result. There may also be danger in injecting one type of prophylactic at certain times after an injection of another type : serious interference may occur with the response to the second injection.

Much work should be done on the subject of immunisation with combined prophylactics. The few results presented here are intended to show that there are more factors in their use than appear to be generally recognised, and to emphasize that synergic effects obtained with certain preparations in certain conditions will not necessarily still occur if any of these conditions are altered. In changed conditions a significant degree of interference may indeed occur instead.

SUMMARY.

The effect of immunisation with a combined prophylactic may depend on the relative proportions of the components, the dosage administered and the immunity state of the population injected. It may also depend on the particular batch of vaccine mixed into a toxoid-vaccine preparation.

Animals immunised with two injections of a combined prophylactic may show significantly lower responses to one component if they have had previous experience of the other.

Synergic effects obtained with a given dose of a combined prophylactic may not occur if the dose of this same preparation, or the proportions of the two components, are altered.

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REFERENCES.

BARR, MOLLIE, AND GLENNY, A. T.—(1949) *J. Hyg., Camb.*, **47**, 107.
BENJAMIN, E., AND WITZINGER, V.—(1912) *Z. Kinderheilk.*, **3**, 73.
CONYBEARE, E. T., AND LOGAN, W. P. D.—(1951) *Brit. med. J.*, **1**, 504.
GLENNY, A. T., HOPKINS, BARBARA E., AND WADDINGTON, HILDA—(1925) *J. Path. Bact.*, **28**, 305.
Idem AND LLEWELLYN-JONES, MONA—(1931) *Ibid.*, **34**, 143.
Idem AND STEVENS, MURIEL F.—(1938) *J. Roy. Army Med. Cps.*, **70**, 308.
Idem AND WADDINGTON, HILDA—(1926) *J. Path. Bact.*, **29**, 118.—(1928) *Ibid.*, **31**, 403.

GREENBERG, L., AND FLEMING, D. S.—(1947) *Canad. J. publ. Hlth*, **38**, 279.—(1948) *Ibid.*, **39**, 131.
HALL, W. W.—(1940) *Ann. int. Med.*, **14**, 565.
LAPIN, J. H.—(1942) *Amer. J. Dis. Child.*, **63**, 225.
LEWIS, J. H.—(1915) *J. infect. Dis.*, **17**, 241.
MACLEAN, I. H., AND HOLT, L. B.—(1940) *Lancet*, ii, 581.
PETERSON, J. C., AND CHRISTIE, A.—(1951) *Amer. J. Dis. Child.*, **81**, 483.
RAMON, G.—(1936) *Rev. Immunol.*, **2**, 415.
Idem AND RICHOU, R.—(1950) *Ibid.*, **14**, 161.
RÖMER, P. H., AND SAMES, T.—(1909) *Z. ImmunForsch.*, **3**, 344.
SCHEIBEL, INGA F.—(1944) *Acta path. microbiol. scand.*, **21**, 130.

INFLUENCE OF CHLORAMPHENICOL AND SPECIFIC ANTISERUM ON THE VARIABILITY OF THE K 12 STRAIN OF *BACT. COLI.*

A. MANTEN* AND D. ROWLEY.

From the Wright-Fleming Institute of Microbiology, St. Mary's Hospital Medical School, London, W.2.

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RECENTLY Voureka (1951) has reported changes in *Bact.* (*Escherichia*) *coli* D 433 induced by treatment with chloramphenicol and specific antiserum. By this means she isolated colonies which differed from the wild type in morphology, sugar fermentations, resistance to antibiotics and in antigenic structure. These changes are far more radical than can be accounted for by current mutational theories.

It thus seemed valuable to repeat Voureka's experiments with the well-known K 12 strain of *Bact. coli* (Lederberg, 1947, 1950) as variations in this strain can be analysed genetically by "re-combination," thereby allowing conclusions to be drawn as to whether the acquired characteristics are truly genotypic mutations or not. Three strains of *Bact. coli* K 12 were used; they were the wild type and the two mutants 58–161 and W677. Several investigators (Lederberg, 1947, 1950; Cavalli and Maccacaro, 1950; Hayes, 1952) have shown that these two mutants will re-combine. Any genotypic alteration in these mutants brought about by chloramphenicol and antiserum should be traceable by a proper analysis of the offspring of the products of re-combination.

EXPERIMENTAL.

The directions given by Voureka were followed as closely as possible.

Ten ml. of broth containing 250 μg./ml. chloramphenicol was inoculated with 0·1 ml. of an overnight broth culture of the bacteria (wild type and the 58–161 and W677 mutants), and after 24 hr. incubation at 37°, to a 1 ml. volume of this an equal volume of antiserum was added. The mixture was then incubated further and every day a small sample was withdrawn for examination. Rigid precautions were taken not to introduce contamination. The antiserum used was a specific K 12 rabbit antiserum with agglutination titre of 1 : 15,000. It was added (*a*) undiluted, and (*b*), as in the experiments of Voureka, diluted to a titre of 1 : 1600 in normal rabbit serum. Controls with normal rabbit serum and distilled water instead of serum were run parallel with the antisera of different concentration.

The samples, withdrawn from the mixtures on successive days, were plated out on nutrient agar plates and on blood plates. Small loopfuls were used so that

* On leave of absence from the National Institute of Public Health, Utrecht, during the tenure of a W.H.O. Fellowship.

no perceptible inhibition arose from the chloramphenicol in the mixtures. Morphologically divergent colonies were streaked for purity on blood plates and the subcultures kept if still different after the second streak.

In order to define any biochemical alterations induced by chloramphenicol and antiserum every subculture was examined for the following properties:

1. Growth requirements of biotin, methionine, threonine, leucine and thiamine, by making streaks on slopes of minimal agar enriched with all these growth factors except one (Davis and Mangioli, 1950).

2. Ability to ferment lactose, maltose, xylose and mannitol. This was determined by inoculating the sub-strains in semi-solid minimal medium enriched with 0·5 per cent casein hydrolysate and 0·1 per cent marmite, to which sugars were added up to 0·5 per cent concentration together with 0·0016 per cent of bromthymol blue as an indicator.

3. Sensitivity to coliphage T5 by streaking the bacteria on blood plates containing coliphage T5.

4. Sensitivity to penicillin, streptomycin, chloramphenicol and sulphathiazole by using nutrient agar slopes in which the drugs had been incorporated in different concentrations.

RESULTS.

Overnight incubation of the three strains with 250 μg./ml. of chloramphenicol resulted in a considerable decrease of viable bacteria in each plate. One-tenth smaller inocula under the same conditions were completely sterilized. Diluting the drug to one-half by addition of an equal volume of water on the second day did not protect those bacteria which had survived the first 24 hr. treatment from being killed during the subsequent 24 hr. In contrast to the behaviour of the bacteria with chloramphenicol alone, the organisms in the tubes to which serum was added on the second day were for the most part still alive on the third and following days. The number of bacteria in these tubes gradually decreased during 6 to 10 days' incubation; thereafter all tubes grew turbid, possibly owing to the emergence of chloramphenicol resistance, or to decomposition of the drug (Smith, Worrell and Lilligren, 1949) during the experiment. This eventual profuse growth invariably occurred earliest in those tubes containing undiluted antiserum.

As described by Voureka, streaks made from the serum-containing tubes any time after the addition of serum showed, after incubation for 48 hr., a variety of colonies ranging in size from minute (0·1 mm.) to those of normal coliform size (2–3 mm.). This disparity was more pronounced in W677 than with 58–161 and the wild type strains.

In agreement with Voureka's observations, it was found that most of the small colony variants, when subcultivated, quickly reverted to normal colonial morphology. Thus out of approximately 250 small colony isolates only 9 maintained their colonial differences for three or more subcultures.

Of these 9 variants 6 were obtained from the W677 strain; 5 of them differed from the parent strain in the small size of their colonies (*ca.* $\frac{1}{2}$–$\frac{1}{3}$ normal); the sixth one was a mucoid variant which was isolated from a sample taken just before turbid growth appeared. From the 58–161 mutant two stable forms were isolated, one of which was a mucoid variant, and the other a very small colony variant (*ca.* 0·1 mm. on blood agar after 24 hr.), which in contrast to the parent 58–161 did not produce any discoloration when grown on blood agar.* Only one stable

variant was isolated from the K 12 wild type. This was a small colony variant similar to that derived from 58–161 above.

On examining these variants for their growth requirements and antibiotic sensitivities, etc., it was found that all those arising from the W677 strain had unaltered characteristics; thus they did not grow in the absence of leucine, threonine and thiamine, and could not ferment maltose, xylose, lactose and mannitol. Sensitivities to coliphage T5, penicillin, streptomycin, chloramphenicol and sulphathiazole were the same as for the parent W677 strain. The only exception was the mucoid variant, which was about twice as resistant to chloramphenicol as the parent strain.

The two variants from 58–161 were rather different. The mucoid form, besides being about twice as resistant to chloramphenicol, was also resistant to T5 coliphage, probably because the mucoid capsule prevented adsorption of bacteriophage. In all other characteristics, such as requirements for biotin and methionine and ability to ferment carbohydrates, the mucoid variant behaved exactly like 58–161. The very small colony variant,* however, did not exhibit any of these biochemical characters; indeed it failed to grow on minimal medium enriched with all five growth factors. Moreover this strain failed to ferment any of the sugars tested, was phage-resistant and was more sensitive to penicillin, chloramphenicol and sulphathiazole than the 58–161 strain.

DISCUSSION.

Chloramphenicol is bactericidal to *Bact. coli* K 12, but on addition of serum, whether normal or antiserum, a small proportion of the bacteria survive the lethal action of the drug. This protective effect of serum is understandable, since the serum proteins probably combine loosely with chloramphenicol and render a part of it inactive (Smith, 1948). The drug concentration is thus lowered partly by adsorption on serum proteins and partly by the dilution consequent on adding the serum.

In agreement with Voureka, we have observed that treatment with chloramphenicol and antiserum (or normal serum) yields a small proportion of cells exhibiting different colonial morphology. With K 12 strains of *Bact. coli* which we have used these alterations are neither so frequent nor so permanent as appeared to be the case with D 433. With the exception of one mucoid variant and two very small colony forms, all our variants possessed the same biochemical characteristics as the parent strains from which they arose. In the case of the two small colony strains further genetic studies appeared difficult if not impossible, as these forms did not grow at all on a medium enriched with all the growth factors required by the W677 and the 58–161 mutants. Since these small colony strains do not grow well on nutrient agar nor even on blood agar, determination of their absolute growth requirements in order to do crossing experiments would probably be very difficult. It seems to us that these variants may be analogous to the small colony variants of *Bact. coli* which have been described by Colwell (1946). In general these latter had much reduced biochemical activities when compared with the parent form; moreover, the high frequency with which they can be made to arise makes it most unlikely that they result from the selection of spontaneous mutants.

* Dr. Voureka examined this variant and reported that it had all characteristics in common with the γ strain which she isolated from *Bact. coli*. D 433.

A few experiments were done to see whether similar variations could be revealed by agents other than chloramphenicol and serum. We found that both streptomycin and penicillin showed the same effects though to a lesser extent ; on the other hand, ultra-violet irradiation was unsuccessful in producing any permanent morphological variation.

SUMMARY.

Colonial variants have been produced in the three *Bact. coli* strains K 12, 58–161 and W677 by treatment with chloramphenicol and antiserum.

Normal serum was shown to be identical with antiserum in producing this effect.

Most of the variants did not differ from the parent strains in the biochemical tests applied.

Two small colony variants which appeared were extremely slow growing and had lost many biochemical activities.

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REFERENCES.

CAVALLI, L. L., AND MACCACARO, G. A.—(1950) *Nature, Lond.*, **166**, 991.
COLWELL, C. A.—(1946) *J. Bact.*, **52**, 417.
DAVIS, B. D., AND MANGIOLI, F. S.—(1950) *Ibid.*, **60**, 17.
HAYES, W.—(1952) *Nature, Lond.*, **169**, 118.
LEDERBERG, J.—(1947) *Genetics*, **32**, 505.—(1950) *J. Bact.*, **59**, 215.
SMITH, J. N., WORRELL, C. S., AND LILLIGREN, B. L.—(1949) *Science*, **110**, 297.
SMITH, R. M.—(1948) *J. Bact.*, **55**, 425.
VOUREKA, A.—(1951) *Lancet*, i, 28.

A STUDY OF ANTIGENIC COMPONENTS OF KIDNEY TISSUE.

A. G. S. HILL* AND B. CRUICKSHANK,

with the technical assistance of

AUDREY CROSSLAND.

From the Departments of Medicine and Pathology, University of Edinburgh, and the Rheumatic Unit, Northern General Hospital, Edinburgh.

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THE phenomenon of " nephrotoxic nephritis," a type of renal damage induced by the injection of an antibody prepared against kidney tissue, has been the subject of many reports. The earliest experiments on these lines were those of Lindemann (1900), but credit for demonstrating nephritic changes clearly attributable to antibodies specific for kidney tissue is generally given to Masugi (1933). In recent years attempts have been made to identify the tissue element in the kidney which stimulates the production of nephrotoxic antibody when introduced into an animal of a different species. Heymann and Lund (1948) reaffirmed an earlier observation (Pearce, 1904) that the cortex of the kidney was very much more active in respect of this antigenic property than the medulla, and Solomon, Gardella, Fanger, Dethier and Ferrebee (1949) discovered that isolated glomeruli could effectively absorb the nephrotoxic antibody from anti-kidney serum. Greenspon and Krakower (1950) found that glomeruli separated from the kidneys of dogs were able to produce a potent nephrotoxic serum when injected into rabbits, while all other components of the renal cortex were ineffective. Krakower and Greenspon (1951) subsequently immunised animals with individual components of glomeruli (separated by sonic vibration), and concluded that the basement membrane of the tufts accounted for most of the antigenic activity of whole glomeruli. The rôle of the glomeruli was again emphasized by Pressman, Hill and Foote (1949), who injected into mice a preparation of anti-mouse-kidney globulin tagged with radio-active iodine and found specific concentration of radio-activity in the glomeruli.

There is therefore good evidence for the importance of the glomeruli and, more specifically, of their basement membrane as antigens in the production of nephrotoxic serum. It is apparent, however, that the antigenic substance is not confined to renal tissue. Both anti-lung (Chikamitsu, 1940) and anti-placenta (Seegal and Loeb, 1946) serum have proved to be nephrotoxic, and anti-lung serum labelled with radio-active iodine has been shown to localise specifically in the kidney as well as in the lung (Pressman and Eisen, 1950).

The concept of a strongly antigenic substance in glomerular basement membrane—a supporting tissue element—seemed to be important not only in helping to elucidate the pathogenesis of experimental nephritis, but perhaps ultimately in relation to the wider problem of the human mesenchymal diseases of unknown

* Present address :—Stoke Mandeville Hospital, Aylesbury, Bucks.

aetiology. It was decided therefore to prepare an anti-kidney serum, to conjugate it with fluorescein, and to apply this "tagged" serum to sections of kidney. The areas of fixation of fluorescent antibody would be expected to be revealed by green fluorescence when such preparations were examined by ultra-violet light. It seemed likely that by the use of this method it would not only be possible to locate the site of the kidney antigen with considerable histological precision, but also to search for tissue with similar antigenic specificity in other organs. The study was subsequently extended to include experiments with anti-glomerulus and anti-lung serum. The results of these investigations have confirmed the antigenicity of glomerular basement membrane, and have shown that an antibody which reacts with this structure reacts also with the basement membrane of renal tubules as well as with a reticular network in the media of vessels. In addition it is believed that the presence of a separate antigen in the cytoplasm of the cortical tubules has been established. A study of other organs, to be reported later (Cruickshank and Hill, 1953), has disclosed a widespread distribution of structures apparently sharing an antigen similar to or identical with the one contained in renal basement membrane.

A method for the detection of antigenic material in tissue cells, in which antibody labelled with fluorescein was employed as a specific histochemical stain, was reported by Coons, Creech, Jones and Berliner (1942), and described in detail in a later paper (Coons and Kaplan, 1950). Fluorescent antibodies have been used to study the uptake by the tissues of injected bacterial polysaccharides (Kaplan, Coons and Deane, 1950; Hill, Deane and Coons, 1950; Schmidt, 1952) and protein antigens (Coons, Leduc and Kaplan, 1951), to identify viruses or rickettsiae in tissues or in tissue culture (Coons, Snyder, Cheever and Murray, 1950; Watson, 1952) and to demonstrate the ACTH-producing cells of the anterior pituitary (Marshall, 1951).

METHODS.

Preparation of antigens and antisera.

Four antisera, prepared in rabbits by the injection of material from rats, were used. The antigens were a suspension of perfused whole rat kidney, a suspension of isolated rat glomeruli, a suspension of perfused rat lung and a preparation made from rat serum. Each rat used as a source of tissue was anaesthetised with ether and perfused with one litre of saline—introduced into the aorta and allowed to escape through an incision in the inferior vena cava. The whole-kidney and lung preparations were made up as 10–15 per cent suspensions in saline and the dosage schedule was 1 ml. twice weekly intraperitoneally for 4–6 weeks. The isolated glomeruli were injected by the same route, a total of 200 mg. (wet wt.) being given over a period of 8 weeks. The glomeruli were separated by a method based on that of Greenspon and Krakower (1950). Antiserum against rat serum proteins was prepared by the method of Heidelberger and Kendall (1935). The serum referred to hereafter as "anti-kidney serum" (as distinct from anti-glomerulus serum) was the preparation obtained by immunization with *whole* kidney homogenate.

Conjugation of serum to fluorescein: preparation, staining and examination of sections.*

The methods used in these procedures, together with the refrigerated microtome cabinet† and the "window" attached to the blade of the microtome, were essentially similar to those

* "Staining" is not a strictly accurate term for a procedure which results in the appearance in sections of an antigen-antibody complex identifiable in ultra-violet light by virtue of a fluorochrome attached to the antibody. For the sake of brevity it is, however, used throughout this paper—to denote both the procedure and the presence of specific green fluorescence in treated tissues.

† Constructed by the Pressed Steel Company, Oxford.

described in detail by Coons and his collaborators (Coons and Kaplan, 1950; Coons *et al.*, 1951). Conjugates were prepared from whole serum and globulin fractions separated from them by ammonium sulphate precipitation; the fluorescent globulin was suspended in the minimal volume of saline and dialysed against buffered saline (pH 7·6). This procedure was adopted simply to achieve a high globulin content in the final preparation, and did not eliminate the non-specific staining which is characteristically produced by untreated conjugates, and which can be prevented (except in a few sites) by absorption with a washed and dried tissue suspension. The identity of the species selected as the source of material for this absorption procedure is probably immaterial when the conjugate is to be used for tracing an antigen artificially introduced into an animal (Coons and Kaplan, 1950). The present experiments, however, were concerned with the detection of antigenic elements in normal rat tissues, and it was considered desirable to treat the conjugates with dried material prepared from an organ of an unrelated species—generally human skeletal muscle. In some experiments a portion of conjugate was subsequently submitted to a second absorption, this time with a suspension of an appropriate *rat* tissue, to remove its *specific* reactivity. This procedure is discussed more fully below.

No fixative was used in preparing tissues for sectioning. Small pieces of each organ to be examined were removed from freshly killed animals, rapidly frozen in a mixture of alcohol and solid carbon dioxide and stored under solid carbon dioxide. The frozen blocks were transferred to the refrigerated microtome cabinet without being allowed to thaw and sections of 6μ thickness cut at a temperature of −18°. Sections were thawed on to gelatinised slides by warming the lower surface of the slide with a finger-tip, dried under a fan and stored at 5°; they were generally stained and examined within 3 days. Sections were treated with fluorescent conjugates for periods of 2–3 hr. at room temperature, and thereafter washed for 10 min. before being mounted in reagent glycerol containing 10 per cent of the same buffer. The investigation did not include any experiments involving the injection of fluorescent globulin *in vivo*; the globulin-fluorescein conjugates were used simply as histochemical reagents on tissue sections.

The light source for the fluorescence microscope was a 250 watt high-pressure mercury arc.* A Corning 5840 ultra-violet filter was used. With this arrangement there was a tendency for the gelatine eyepiece filter (Wratten 2B) to fluoresce faintly; this was remedied by placing a glass cell containing 2 per cent sodium nitrite below the gelatine disc.

Tests for specificity of fluorescent staining.

The specificity of the green fluorescence in sections which had been exposed to fluorescein-globulin conjugates prepared from anti-kidney serum was tested in several ways. It was necessary to establish that the staining was dependent on a factor present in immune serum but not in normal rabbit serum, and that this factor was active in the absence of the fluorescein "label." This was accomplished by the exposure of sections to the unlabelled antiserum and to normal rabbit serum respectively, and thereafter treatment of both sections with fluorescent globulin prepared from the antiserum. Fluorescent staining failed to develop in the first of these sections (indicating that the reactive groups of the antigen had been specifically bound by the unlabelled antibody), but appeared as usual in the second. As a further check on the absence of the specific factor from the serum of a non-immunised rabbit, a third section was treated with a fluorescein-globulin conjugate prepared from such serum and was found to show no staining. It was also found that a homogenate of rat kidney would absorb the antibody from the anti-kidney conjugate, with resulting loss of its capacity to produce staining, but that similar absorption with rat liver (which contained little stainable material) or with kidney from an unrelated species (pig) was ineffective. The results of absorption experiments with rat lung, of inhibition by pre-treatment with unconjugated anti-lung serum and of other control experiments will be described later.

In the inhibition experiments with unlabelled serum trials were made of various lengths of exposure both to serum and, thereafter, to conjugate; in general, pre-treatment for 3 hr. and staining for 1–1½ hr. gave clear-cut results. In the absorption experiments 1 part of a wet packed organ suspension was mixed with 3 parts of fluorescent conjugate; after 1 hr. the suspension was removed by centrifugation and replaced for a second hour with fresh homogenate in the same proportion.

* Mazda ME/D 26.

RESULTS.

The patterns of staining produced in rat kidney by fluorescent globulin from three antisera—anti-kidney, anti-glomerulus and anti-lung—are summarised in the Table, which also shows the results of pre-treatment with unlabelled sera, and of absorption of the anti-whole-kidney conjugate with organ suspensions.

TABLE.—*Staining Reactions in Rat Kidney.*

Conjugate.	Tissue element. Glomerular basement membrane.	Tubular basement membrane.	Tubular cytoplasm (cortex).	Media of vessels.	Figure.
Anti-rat-kidney globulin (AKG)	+	+	+	+	1, 2, 3
Anti-rat-glomerulus globulin (AGG)	+	+	N	+	4, 5
Anti-rat-lung globulin (ALG)	+	+	N	+	
AKG absorbed with rat kidney	N	N	N	N	
" " with rat lung	N	N	+	N	6
" " with rat liver	+	+	+	+	
" " with pig kidney	+	+	+	+	
AKG after pre-treatment with unlabelled homologous serum	N	N	N	N	
AKG after pre-treatment with unlabelled anti-lung serum	N	N	+	N	
AKG after pre-treatment with unlabelled normal rabbit serum	+	+	+	+	
Normal rabbit globulin	N	N	N	N	

(+ = Specific green fluorescence. N = No specific fluorescence.)

DESCRIPTION OF PLATES.

FIG. 1–6 are photomicrographs of sections of normal rat kidney after exposure to fluorescein-labelled antibody globulin. The lightest areas represent the bright green fluorescence which indicated specific fixation of fluorescent antibody by the homologous tissue antigen: the histological topography in areas where no specific reaction occurred is visible as a darker image, by virtue of the faint bluish auto-fluorescence of the tissues. The sections in Fig. 7 and 8 were photographed under an ordinary light microscope.

FIG. 1.—Treated with anti-kidney globulin. Specific fluorescence is present in the glomeruli and in the cytoplasm of the convoluted tubules. Fluorescence is also present in the tubular basement membrane, but this feature is more easily seen in the high-power view (Fig. 3). × 160.

FIG. 2.—High-power view of a glomerulus from the preparation illustrated in Fig. 1. The basement membrane of the tuft and of Bowman's capsule is distinctly seen as a single bright line; staining is also present in the cytoplasm of epithelial cells. × 480.

FIG. 3.—High-power view of convoluted tubules from the preparation illustrated in Fig. 1 and 2. Specific fluorescence is present in the basement membrane and in the epithelial cytoplasm; no reaction is visible in the nuclei. × 550.

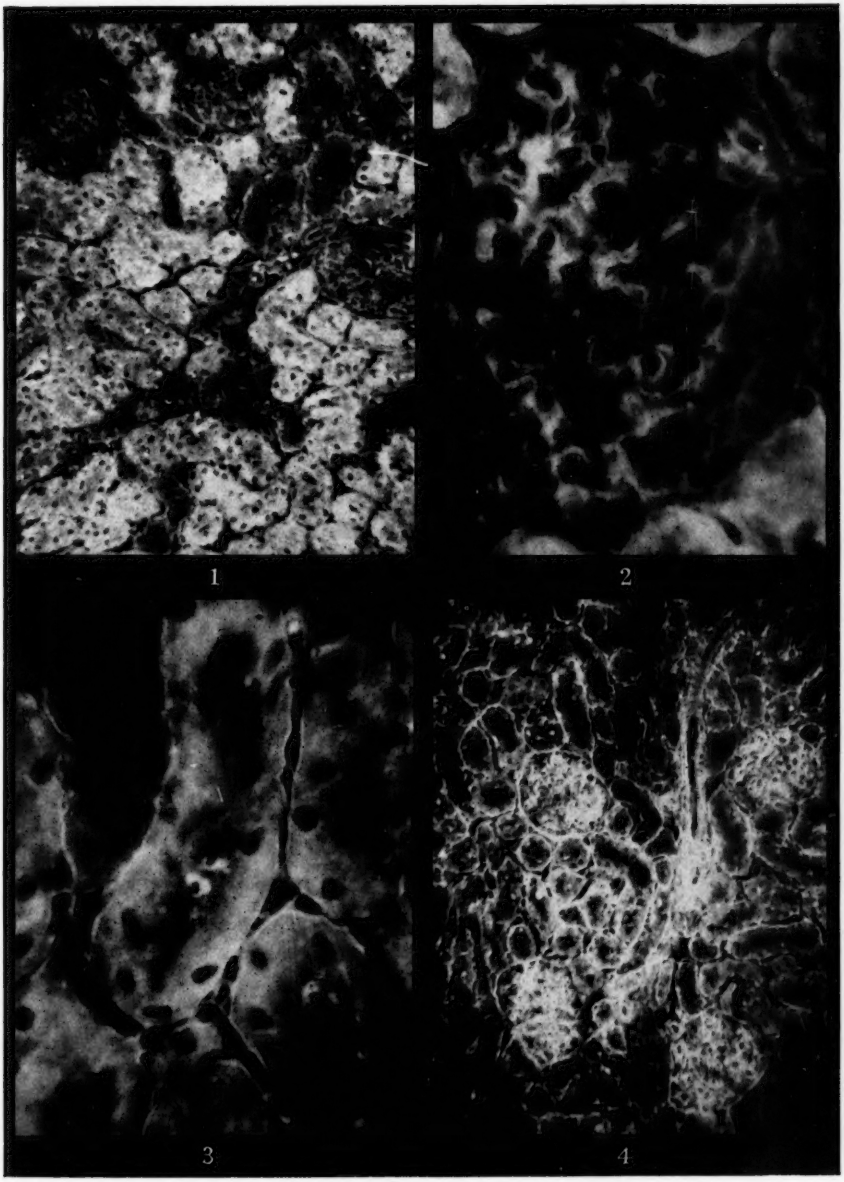
FIG. 4.—Treated with anti-glomerulus globulin. Specific fluorescence is present in the glomeruli and in the basement membrane of the convoluted tubules, but (in contrast to Fig. 1) the tubular epithelium is unstained. × 140.

FIG. 5.—High-power view of convoluted tubules from the preparation illustrated in Fig. 4. The presence of staining in basement membrane and its absence in the tubular cytoplasm is clearly seen (compare with Fig. 3). × 550.

FIG. 6.—Treated with anti-kidney globulin which had been absorbed with a homogenate of rat lung. Fluorescence in the tubular cytoplasm is similar to that seen in Fig. 2, but the glomeruli are unstained. × 160.

FIG. 7.—Section of kidney (stained with haematoxylin and eosin) from a rat killed 4 hr. after an intravenous injection of anti-glomerulus globulin. The two glomeruli show excessive cellularity (an appreciable number of polymorphs were identifiable under the microscope) and a considerable reduction in the number of red cells. × 300.

FIG. 8.—Section of kidney (stained with haematoxylin and eosin) from a rat killed 16 days after an injection of anti-kidney serum. The glomerulus shows generalised thickening of basement membrane with patchy necrosis and markedly reduced vascularity. A cast can be seen in an adjacent tubule. × 300



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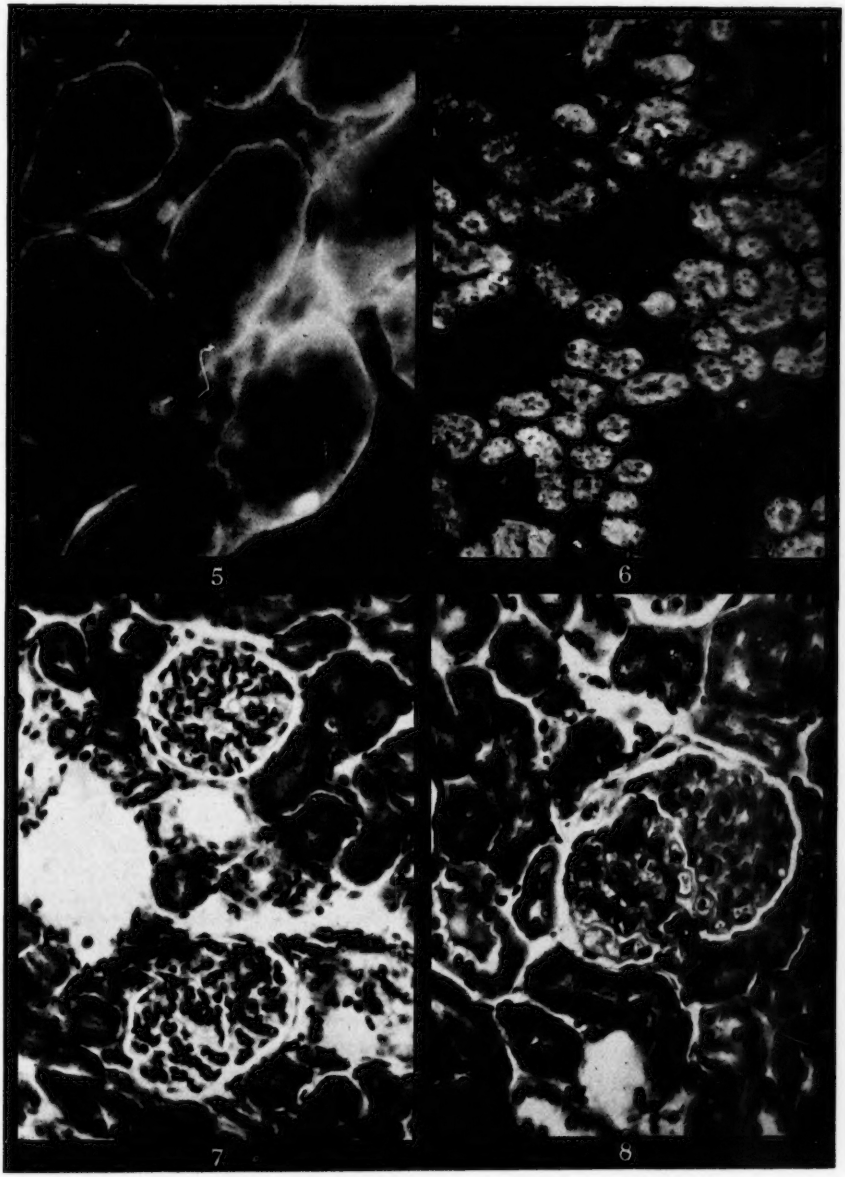

Hill and Cruickshank.

The anti-kidney preparation reacted with basement membrane, both glomerular and tubular, with the cytoplasm of tubules and with a reticular structure in the media of vessels (Fig. 1–3). In the glomerular tuft a single bright green line outlined the capillary lumina. The endothelium was unstained, but the cytoplasm of the epithelium on the outer surface of the membrane had a fainter green colour. No second membrane was seen beneath this epithelium. The basement membrane of the capsule was also seen as a green line, thinner than that of the tuft. All the cortical tubules showed specific fluorescence, both in their basement membrane and in the cytoplasm of their epithelium : in the medulla, however, staining in these two sites was only occasionally present. It was not possible to identify the few medullary tubules with which the conjugate reacted. Capillaries elsewhere than in the tuft showed the same single fluorescent membrane outside the endothelium. Staining in the media of larger vessels took the form of a distinct reticular pattern surrounding the plain muscle fibres ; the elastic laminae were prominent by virtue of their natural bright bluish-white fluorescence and were readily distinguished from this specific green-staining network. No nuclear staining was seen.

The anti-glomerulus preparation reacted with glomerular and tubular basement membrane and with reticulin in the media of vessels in exactly the same way as the anti-kidney preparation ; unlike the latter it did not, however, react with the tubule cell cytoplasm (Fig. 4 and 5). The anti-lung preparation produced staining which was identical in pattern with that of sections stained with anti-glomerulus globulin but lower in intensity.

The specificity of the two types of antisera—anti-kidney on the one hand and anti-glomerulus or anti-lung on the other—was confirmed by the inhibition experiments. Thus unconjugated anti-glomerulus and anti-lung sera were able to inhibit the staining of basement membrane by anti-kidney conjugate but not the staining of tubule cells, whereas substitution of unconjugated anti-kidney serum resulted in inhibition of staining in all sites. The results of absorption experiments were equally consistent. Whole kidney suspension effectively removed all the staining properties of anti-kidney conjugate ; lung suspension, on the other hand, absorbed out only the basement membrane staining factor (Fig. 6). In all these experiments staining of reticulin in the media of vessels was retained or lost in parallel with basement membrane staining.

Apart from the serum inhibition and absorption experiments and controls with fluorescent normal rabbit globulin the specificity of the staining reactions was checked in various ways. It seemed necessary to eliminate the possibility that staining was simply due to a reaction between serum protein in the tissues and the corresponding antibody in the conjugates. The very weak precipitin reaction which the anti-kidney serum gave with rat serum made this unlikely, but it was thought desirable to prepare a fluorescent conjugate from a potent antiserum against rat serum proteins. This preparation produced some faint fluorescence in sections of rat kidney, but the pattern did not resemble that produced by the anti-organ preparations. The anti-kidney preparation strongly agglutinated rat erythrocytes, but absorption of the agglutinin from the conjugate left its reactivity with kidney tissue unchanged. Finally, to investigate the species specificity of the reaction, anti-rat-kidney conjugate was applied to mouse, guinea-pig, pig, rabbit and human kidney sections. Except in the case of the mouse no reaction occurred : in mouse kidney staining similar to that in the

rat was produced. An immunological cross-reaction between rat and mouse kidney has been noted previously (Pressman, 1949).

The ability of the anti-rat glomerulus serum to produce nephritis was established in an experiment based on the observations of Heymann and Hackel (1952). Rats weighing 50–65 g. were given a single intravenous injection and killed 4 hr. later. Whole anti-glomerulus serum (0·75 ml.) was used in one animal, globulin from the same serum (0·5 ml. of a solution containing 1·7 g./100 ml.) in 3 more, and normal rabbit serum (0·8 ml.) in 2 control rats. In the kidneys of all 4 animals injected with anti-glomerulus serum or globulin there was a considerable increase in the cellularity of the glomeruli, together with decreased vascularity, swelling of endothelium, patchy thickening of the basement membrane in some tufts and blurring of the outline of capillaries in others (Fig. 7). No changes were seen in the tubules except for distension with albuminous fluid in one animal. No pathological changes were found in the kidneys of the rats injected with normal rabbit serum. In other experiments massive proteinuria (up to 1·9 g./100 ml.) was induced by injections of anti-kidney or anti-glomerulus globulin ; in a section from a rat killed 16 days after an injection of the former there was generalised thickening of the basement membrane in the glomeruli with some patchy necrosis and markedly reduced vascularity (Fig. 8).

DISCUSSION.

The earlier experiments in this investigation, in which fluorescein-conjugated anti-kidney globulin was applied to sections of rat kidney, showed the feasibility of using such a conjugate as a histochemical reagent for visualising antigenic tissues. This reagent reacted, however, both with basement membrane (glomerular and tubular) and with the cytoplasm of tubular epithelium ; the nephrotoxic activity of the serum from which it was prepared could therefore not necessarily be attributed solely to an antibody against basement membrane. In order to establish the significance of such an antibody it was clearly essential to prepare a nephrotoxic serum which *in vitro* would react only with basement membrane. Substitution of isolated glomeruli for whole kidney suspension as the immunising material provided a serum which reacted in this way and was also capable of inducing nephritis when injected *in vivo*. Whether or not fixation of antibody by the basement membrane of tubules as well as by that of the glomeruli plays a significant part in the mechanism of this type of experimental nephritis must remain uncertain : early histological changes in glomerular basement membrane are well recognised, but corresponding changes in the basement membrane of tubules have not been described. The results now reported indicate that the epithelium of the cortical tubules contains an antigen distinct from the one which is present in basement membrane, but that serum may possess nephrotoxic activity in the absence of antibody against this tubule antigen. The anti-lung serum resembled the anti-glomerulus preparation in its reaction with kidney tissue sections, an observation consistent with the evidence, quoted in the introductory section of this report, that an identical or similar antigen is to be found in organs other than the kidney.

It would clearly be desirable to know more about the identity of the basement membrane antigen. Its chemical nature is largely speculative, though the reaction of glomerular and tubular basement membrane in the periodic acid—Schiff procedure suggests that it may contain a polysaccharide. This suggestion

is consistent with an observation made by Cole, Cromartie and Watson (1951), who prepared a tryptic digest of kidney suspension and found that it contained a soluble substance capable of absorbing kidney-damaging antibody from nephrotoxic serum. They suggested that the kidney antigen may be a complex molecule containing protein in combination with a polysaccharide or lipid.

It is well known that the immunological activity of anti-kidney serum measured *in vitro* by its reactivity with a supernate from a homogenate of kidney tissue bears little relation to its ability to produce nephritis (Smadel, 1936). If it is correct to conclude that such serum is nephrotoxic by virtue of its ability to react with renal basement membrane this observation is not surprising; the activity of serum assessed *in vitro* against such a supernate may well be independent of its content of basement membrane antibody, for the antigen in basement membrane is probably insoluble in aqueous media (Cole *et al.*, 1951). It is obviously an advantage to be able to demonstrate a reaction between a tissue antibody and an antigenic component of an organ, which may only account for a small proportion of the total bulk of the organ and which may well not be represented in a supernate from homogenised material. The introduction of antibodies labelled with a radio-active tracer into this type of work represented a notable advance. Pressman *et al.* (1949), using this type of preparation, were able to demonstrate fixation of anti-kidney globulin in the glomeruli, but not, apparently, to identify the exact site of the reaction within these structures, nor to demonstrate any reaction with tubular basement membrane. More precise histological identification of the site of activity of anti-kidney antibodies has proved possible in the present investigation, and the results obtained suggest that the fluorescein labelling technique may prove generally useful in the field of tissue antibody reactions.

SUMMARY.

The properties of antibodies prepared against homogenates of whole kidney, isolated glomeruli and lung from normal rats have been investigated. Fluorescein-labelled globulin from each of these antisera has been applied to unfixed sections of normal rat kidney and the sites of specific fixation determined by fluorescence microscopy.

Globulin from the anti-whole-kidney serum reacted with basement membrane in the glomeruli and convoluted tubules and also with the cytoplasm of the tubular epithelium. Antibody globulin prepared by immunisation with lung tissue or glomeruli also reacted with basement membrane, but failed, on the other hand, to react with tubular epithelium. It is concluded that kidney tissue contains at least two distinct antigens—one in basement membrane and the other in the cytoplasm of the convoluted tubules. By the injection of the anti-glomerulus serum into rats it has been shown that an antibody specific for the first of these antigens is capable of producing nephritis.

By the use of fluorescein-labelled antibodies as histochemical reagents it has been possible to locate these antigenic elements in normal renal tissue with considerable precision, and it is suggested that the method may be useful in the study of antibodies against other tissues.

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REFERENCES.

CHIKAMITSU, H.—(1940) *Folia endocrin. jap.*, **16**, 85. Quoted by Pressman, D. (1951) *J. Allergy*, **22**, 387.

COLE, L. R., CROMARTIE, W. J., AND WATSON, D. W.—(1951) *Proc. Soc. exp. Biol., N.Y.*, **77**, 498.

COONS, A. H., CREECH, H. J., JONES, R. N., AND BERLINER, E.—(1942) *J. Immunol.*, **45**, 159.

Idem AND KAPLAN, M. H.—(1950) *J. exp. Med.*, **91**, 1.

Idem, LEDUC, E. H., AND KAPLAN, M. H.—(1951) *Ibid.*, **93**, 173.

Idem, SNYDER, J. C., CHEEVER, F. S., AND MURRAY, E. S.—(1950) *Ibid.*, **91**, 31.

CRUICKSHANK, B., AND HILL, A. G. S.—(1953) *J. Path. Bact.*, in press.

GREENSPON, S. A., AND KRAKOWER, C. A.—(1950) *Arch. Path.*, **49**, 291.

HEIDELBERGER, M., AND KENDALL, F. E.—(1935) *J. exp. Med.*, **62**, 697.

HEYMANN, W., AND HACKEL, D. B.—(1952) *J. Lab. clin. Med.*, **39**, 429.

Idem AND LUND, H. Z.—(1948) *Science*, **108**, 448.

HILL, A. G. S., DEANE, H. W., AND COONS, A. H.—(1950) *J. exp. Med.*, **92**, 35.

KAPLAN, M. H., COONS, A. H., AND DEANE, H. W.—(1950) *Ibid.*, **91**, 15.

KRAKOWER, C. A., AND GREENSPON, S. A.—(1951) *Arch. Path.*, **51**, 629.

LINDEMANN, W.—(1900) *Ann. Inst. Pasteur*, **14**, 49.

MARSHALL, J. M.—(1951) *J. exp. Med.*, **94**, 21.

MASUGI, M.—(1933) *Beitr. path. Anat.*, **91**, 82.

PEARCE, R. M.—(1904) *Univ. Pa. med. Bull.*, **16**, 217.

PRESSMAN, D.—(1949) *J. Immunol.*, **63**, 375.

Idem AND EISEN, H. N.—(1950) *Proc. Soc. exp. Biol., N.Y.*, **73**, 143.

Idem, HILL, R. F., AND FOOTE, F. W.—(1949) *Science*, **109**, 65.

SCHMIDT, W. C.—(1952) *J. exp. Med.*, **95**, 105.

SEEGAL, B. C., AND LOEB, E. N.—(1946) *Ibid.*, **84**, 211.

SMADEL, J. E.—(1936) *Ibid.*, **64**, 921.

SOLOMON, D. H., GARDELLA, S. W., FANGER, H., DETHIER, F. M., AND FERREBEE, J. W.—(1949) *Ibid.*, **90**, 267.

WATSON, B. K.—(1952) *Proc. Soc. exp. Biol., N.Y.*, **79**, 222.

THE ACTION OF PENICILLIN AND TERRAMYCIN ON PNEUMOCOCCAL INFECTION IN NORMAL AND LEUCOPENIC RABBITS.

J. L. GOWANS.

From the Sir William Dunn School of Pathology, Oxford.

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AN antibiotic is selected for trial against experimental infections when it combines low toxicity for animals with high antibacterial activity *in vitro*, although these properties do not always ensure that it will be therapeutically effective. With penicillin and the newer antibiotics used in clinical medicine, the range of activity *in vitro* (but not always the degree of activity) correlates well with their performances *in vivo*. There are other antibiotics, however, where such a correlation does not exist. For example, micrococcin is highly active against *Str. pyogenes in vitro*, but fails to protect mice infected with an inoculum of this organism which is easily dealt with by penicillin (Heatley, Gowans, Florey and Sanders, 1952). Similarly, helvolic acid (Florey and Jennings, 1949) did not control staphylococcal infections in mice satisfactorily, although treatment had conferred on the blood strong inhibitory powers against the staphylococcus. These discrepancies suggest that an essential property of a successful chemotherapeutic agent may be the ability to kill bacteria, since micrococcin and helvolic acid are predominantly bacteriostatic *in vitro*. On the other hand, the effectiveness of chloramphenicol, aureomycin and terramycin, which are held by many to exert a bacteriostatic action at the concentrations likely to occur *in vivo*, does not at first sight support this idea.

Penicillin will exert a rapid lethal action on susceptible micro-organisms *in vitro* under conditions that in the absence of penicillin would allow bacterial growth (Hobby, Meyer and Chaffee, 1942), although a variable number of " persisters " escape destruction by even high concentrations of the drug (Bigger, 1944 ; Spicer and Blitz, 1948). Chloramphenicol, aureomycin and terramycin will also, under certain experimental conditions, kill the majority of a population of susceptible bacteria, but a small proportion will survive and resume growth if the drug becomes inactivated on prolonging the incubation (Spicer, 1950 ; Hobby, Lenert, Pikula, Kiseluk and Hudders, 1950). The observation that such surviving organisms resume growth has tended to obscure the importance of the initial bactericidal action of chloramphenicol, aureomycin and terramycin.

The speed with which penicillin causes bacteria to disappear from the lesions of experimental and human infections suggests that it can exert a direct bactericidal action *in vivo*. Eagle, Fleischman and Musselman (1950) demonstrated such an action in mice infected in the muscle of the thigh with streptococci and pneumococci. Systemic treatment with penicillin was started immediately after infection, and viable counts performed on homogenates of the excised muscle showed that the organisms were destroyed as rapidly *in vivo* as they had been *in vitro*. However, the conditions in these experiments were relatively simple ; the drug was acting against a small number of bacteria and before the development of an inflammatory lesion.

There is both clinical and experimental evidence that penicillin can be therapeutically effective in the absence of specific immunological responses (*e.g.*, Rantz, Boisvert and Spink, 1946; Harrison, 1946), but there are conflicting views on the part played by phagocytes.

Levaditi (1945) concluded that phagocytosis played an essential part in sterilizing the peritoneal exudate of mice infected with staphylococci and treated subcutaneously with penicillin. On the other hand, Jennings and Florey (1949) did not observe any increased phagocytosis to account for the disappearance of haemolytic streptococci from the peritoneal cavity of mice treated systemically with penicillin.

Skinsnes (1948) observed no more phagocytosis in sections of skin from rats and guinea-pigs infected intradermally with Type 1 pneumococcus and treated with penicillin than in the control animals. Skinsnes also showed that penicillin could bring about a rapid reduction in the number of pneumococci in guinea-pigs, the circulating leucocytes of which had been reduced to 1000–1900 cells per c.mm. by intravenous injections of nitrogen mustard. In the untreated leucopenic guinea-pigs infected with pneumococci there was little inflammatory reaction in the skin and almost no phagocytosis; in the treated leucopenic animals the inflammatory response was even less than in the controls but "no free cocci were present after ten hours' treatment."

These experiments with guinea-pigs do not provide a rigorous test of the bactericidal action *in vivo* of penicillin. The guinea-pig is relatively resistant to infection with pneumococci, and the leucocyte counts in the leucopenic animals were too high to rule out a contribution by phagocytes. It is not stated if the lesions became sterile or how long the leucopenic animals survived.

In summary, although the clinical and experimental evidence is in favour of the view that penicillin can exert a bactericidal action *in vivo*, it is still not certain whether penicillin alone can sterilize infections or whether the co-operation of leucocytes is essential for the complete elimination of infecting bacteria.

The part played by immune bodies and leucocytes in the cure of infections by chloramphenicol, aureomycin and terramycin is not known. Similarly a bactericidal action *in vivo* by the newer antibiotics has not been conclusively demonstrated although, as with penicillin, the speed with which treatment eliminates bacteria from human and experimental infections suggests that it does occur.

EXPERIMENTAL.

The present experiments were designed to investigate the mode of action of penicillin and terramycin on an otherwise fatal pneumococcal skin infection in rabbits. In order to assess the contribution of the body defences rabbits were infected after their granulocytes had been to a large extent eliminated by repeated injections of benzene. The immediate problems were to see if large doses of penicillin or terramycin administered systemically could sterilize the leucocyte-free lesions, and to determine the fate of the cocci in the treated animals by histological study of the skin.

Ideally each experiment comprised 4 rabbits: (*a*) normal infected untreated, (*b*) leucopenic infected untreated, (*c*) normal infected, treated with antibiotic, (*d*) leucopenic infected, treated with antibiotic. Rabbit (*a*) served to check the virulence of the pneumococcus; rabbit (*b*) was usually omitted since so few satisfactory leucopenic animals were obtained.

The infection.

The extensive skin lesion and septicaemia caused by the intradermal injection of virulent Type 1 pneumococcus in rabbits is particularly suitable for investigating the action of anti-

biotics since the progress of the infection can be watched by the naked eye ; it is invariably fatal in untreated animals, and the fate of the cocci can be followed by histological and bacteriological studies of the infected skin.

The virulence of the Type 1 pneumococcus (CN 33 strain of the Wellcome Laboratories) was maintained by mouse passage every three weeks, and a lethal dose for mice was regularly less than 50 organisms. The virulence was always tested in mice before an experiment on rabbits was performed and it was found to run parallel in the two species.

Rabbits of either sex, weighing 1·5–2·5 kg., were infected high up on both flanks with 0·2 ml. of an undiluted 6-hr. culture in serum broth. The lesion on one side was employed for histological study ; that on the other side was excised and cultivated after treatment to determine if any viable organisms remained.

Leucopenia.

Leucopenia was produced in rabbits by the daily subcutaneous injection of 2 ml. of a 1 : 1 mixture of benzene and arachis oil /kg. body wt. (Selling, 1916). The injections were made alternately into the left and right groins. Rich and McKee (1934) stated that the benzene method of producing a profound leucopenia was extremely " capricious," and this was amply confirmed in the present work. Of 57 animals given benzene, 6 were abandoned owing to skin sloughs, 9 died suddenly after an injection, and 15 died without the leucocyte count falling to the desired level. Experiments were begun on the remaining 27 rabbits. Many of these died before an experiment could be completed. In others the counts rose after the initial fall and the experiments had to be discontinued. No animal whose total daily white-cell count persisted below 500 cells/c.mm. survived longer than 5 days ; the average survival time of 16 such leucopenic rabbits was 50 hr.

Rich and McKee stated that leucocytes fail to migrate into infected skin when the total white-cell count is less than 250 cells/c.mm. In the present experiments leucopenic animals were infected when the total white-cell count was less than 250 cells/c.mm. or when a differential count showed fewer than 150 granulocytes/c.mm. The counts fell to these levels after 6–12 daily injections of benzene (average in 27 rabbits, 8 injections) ; no further injections were then given. Some granulocytes were always present in the skin of the infected antibiotic-treated leucopenic rabbits, even those with leucocyte counts persisting below 250 cells/c.mm. As the extent of this infiltration could not be predicted from the leucocyte counts, an experiment was finally judged as satisfactory on histological grounds. If more than a scattering of granulocytes was present in stained sections of skin, or aggregates of such cells were seen, the experiment was rejected.

A *Ps. pyocyanea* septicaemia developed terminally in some of the animals surviving longest. This was presumably caused by secondary invasion from the gut and it made any bacteriological studies on the local lesions and the blood impossible. If a leucopenic rabbit developed patches of skin purpura and a watery blood-stained diarrhoea it was always killed and examined, for these signs were usually accompanied by secondary bacterial contamination of the tissues.

In an attempt to treat the profound anaemia and dehydration developing in rabbits poisoned with benzene, transfusions of 20 ml. stored whole blood and subcutaneous injections of 20 ml. glucose-saline were started after infection with the pneumococcus and continued twice daily. These measures resulted in some general improvement in the animals' condition but they did not prolong life.

Of the 27 experiments on treated leucopenic rabbits, 7 were satisfactory and are recorded in the Table. A limited amount of information, mainly histological, was obtained from the remaining 20.

Bacteriological methods.

The success of treatment could not be judged by the survival of the leucopenic animals since they quickly died from the effects of the benzene. The criteria of cure were therefore bacteriological. The treated rabbits were killed about 12 hr. after the last dose of antibiotic (or as long as possible after the last dose in the case of some of the very sick leucopenic animals), and blood, oedema fluid and a homogenate of the skin lesions were cultivated. Penicillinase was added to media inoculated with material from penicillin-treated animals, and it was assumed that dilution and prolonged incubation would allow the detection of surviving organisms in material from animals treated with terramycin.

TABLE—*Results of Treating a Pneumococcal Skin Infection in Leucopenic Rabbits with Penicillin and Terramycin.*

Rabbit No.	Number of benzene injections.	Leucocytes on 1st and 2nd days after infection. (Cells/c.mm.) Total.	Polymorphs.	Drug.	Duration of treatment in hours.	Bacteriology of tissues. Skin lesion.*	Blood.	Time of death in hours after last dose of antibiotic. Animal killed (K) or Died (D).	
1	9	380	80	Penicillin	23	Pneumococci, about 1000 colonies per plate	Sterile	14½	K
		280	10						
2	6	420	60		34	Sterile	,,	6	K
		230	0						
3	7	200	30		42	,,	,,	6	K
		90	20						
4	10	130	40	Terramycin	25	Pneumococci about 200 colonies per plate	,,	3	D
		20	0						
5	11	90	0		27	Pneumococci, about 1000 colonies per plate	,,	½	D
		10	0						
6	12	670	60		66	Sterile	,,	18	K
		620	120						
7	10	330	100		50	,,	,,	11	K
		30	0						

* Homogenate of lesion spread on blood agar plates.

Cultures of blood and oedema fluid.—Each morning, about 12 hr. after the last dose of antibiotic, 0·1 ml. blood was obtained from a marginal ear vein and spread on a blood-agar plate. Heart blood from dead animals was cultivated on blood agar, and about 1 ml. was also cultivated in serum broth and subsequently plated on blood agar. Oedema fluid, collected in a pipette from the subcutaneous tissues of dead animals, was cultivated in the same way as heart blood. All the plates were incubated for 72 hr.

Bacteriology of the local lesion.—The surface of the skin was lightly seared with a microburner. A flap of skin containing the lesion was turned back, and the subcutaneous tissue and deeper layers of the dermis were dissected off and dropped into a sterile screw-capped bottle. The excised tissue was cut up with scissors and ground with 10 ml. peptone water in a sterile Potter's homogenizer. Single drops (about 0·02 ml.) of the homogenate were spread on five blood agar plates and the rest, including any remnants of tissue, was distributed between ten bottles each containing 20 ml. serum broth, which was subsequently plated on blood agar. All the plates were incubated for 72 hr. A drop of homogenate from a 6-hr. lesion of an untreated normal or leucopenic animal always produced a confluent growth of pneumococcus.

Histology.

Small pieces of skin were excised under anaesthesia at various times after infection. At death the whole lesion from one side of the rabbit was cut out and fixed for 24 hr. in Carlton's fixative. Sections, cut at 5μ, were stained with Weigert's iron haematoxylin followed by the Claudius modification of Gram's stain.

Treatment.

Injections of the antibiotic were given at 10 a.m., 4 p.m. and 10 p.m. each day, beginning 6 hr. after infection.

Penicillin.—Penicillin in doses of 12,000 units/kg. was administered in aqueous solution, half each dose being given intravenously and half intramuscularly. A double dose was given at the start of treatment and at 10 p.m. each night.

Terramycin.—The commercial preparation of terramycin hydrochloride buffered with sodium glycinate was given intravenously in a solution containing 5 mg. terramycin/ml. This solution caused some discomfort on injection into an ear vein. Each dose contained 40 mg. terramycin/kg. body wt.

RESULTS.

Normal, Infected, Untreated Rabbits.

Six hours after infection the lesion was a raised red area measuring about 1·5 × 2·5 cm. and the blood culture was always positive. From 6 to 24 hr. the lesion increased rapidly. A raised red haemorrhagic zone about 4 cm. wide spread to the mid-ventral line, where the oedema fluid collected in a pendulous sac.

Histologically the main features were the rapid proliferation of cocci, and the inability of the violent inflammatory reaction to localize the infection. A continuous thick band of granulocytes accumulated in the dermis except at the lower spreading edge of the lesion, where the oedema fluid was carrying down an almost cell-free suspension of pneumococci. Granulocytes containing morphologically normal Gram-positive cocci were numerous in the more compact areas of skin; in fact, where the inflammatory cells were most numerous, relatively few extracellular cocci were seen. This phagocytosis in no way kept pace with the multiplication of the organisms, which spread freely into the muscle layers deep to the skin, and in the advancing mass of oedema fluid. The oedema fluid could be readily pipetted off from the subcutaneous tissues when the lesion was dissected. It had an oily consistency, contained a few degenerating cells, and was crammed with cocci. There were conspicuous haemorrhages throughout the infected skin, dilated blood-vessels and lymphatics, and almost no fibrin.

The animals died 27–50 hr. after infection (average in 8 rabbits, 37 hr.).

Leucopenic, Infected, Untreated Rabbits.

The blood cultures were always positive 6 hr. after infection. Four leucopenic rabbits with total leucocyte counts of 350, 210, 280, and 100 cells/c.mm. died respectively at 20, 12, 29½ and 25½ hr. after infection.

The lesions in this group of rabbits were remarkable for the lack of inflammatory changes. Macroscopically, little could be seen at 6 hr.; later, a pale blue discoloration of the skin appeared. Terminally the lesions were about the same size as those in normal animals, but there was a conspicuous absence of erythema and much less oedema. Purpura of the skin sometimes developed late in the infection.

The bacteria grew exuberantly in a continuous layer in the skin. They tracked between the dermis and the subcutaneous muscle, invading the latter and leaving enormous numbers of cocci growing in clumps around the blood-vessels in the skin. The cellular response was negligible. A few scattered granulocytes and some mononuclear cells (presumably macrophages) were seen, but there was no phagocytosis.

Normal, Infected, Treated Rabbits.

Four rabbits were treated with penicillin, 2 for 24 hr. and 2 for 48 hr.; 5 were treated with terramycin, 2 for 24 hr. and 3 for 48 hr. The pathological and bacteriological findings in the animals treated with penicillin and terramycin were identical.

When treatment was started blood cultures were always positive and large numbers of cocci were present in the skin. The second blood culture taken about 12 hr. after the initial dose of antibiotic was always negative, and succeeding blood cultures remained negative. Macroscopically, the spread of the lesions was quickly arrested by the treatment, although some oedema fluid continued to seep downwards to form a swelling in the ventral abdominal wall. The intensity of colour increased until after about 18 hr. of treatment there was a well-marked red area with a paler erythematous surround. At no time did the treated animals appear ill.

Microscopically the main feature was localization of the infection; the granulocytes became aggregated into discrete foci and the bacteria did not invade the muscle under the dermis. The number of extracellular cocci steadily diminished, but there was no more phagocytosis by granulocytes in the lesions of the treated than of the untreated animals. The cocci persisted longest in the foci of disintegrating granulocytes and in the oedema fluid.

After 24 hr. of treatment there were still many extracellular cocci, and homogenates of the lesions grew a variable number of discrete colonies of pneumococci on blood agar; after 48 hr. extracellular organisms had disappeared and the lesions were sterile. At this time many macrophages were seen among the fragmenting granulocytes and many were filled with well-stained Gram-positive bacterial debris, often recognizable as the remains of diplococci.

One each of the rabbits treated with penicillin and terramycin for 48 hr. was killed several days after the end of treatment when surviving organisms might have resumed growth. The lesions were sterile.

Leucopenic, Infected, Treated Rabbits.

Blood cultures were positive 6 hr. after infection and became persistently negative after the first dose of penicillin or terramycin. Macroscopically the

lesions remained pale and inconspicuous, their appearance contrasting with the well-marked reaction in normal treated or untreated animals. A ring of punctate haemorrhages sometimes appeared at the edge of the lesions during the course of treatment and a little pitting oedema was usually present in the abdominal wall.

The results of experiments with 7 leucopenic rabbits are summarized in the Table. These experiments were regarded as satisfactory since there was no secondary bacterial contamination of the blood or skin, and only a few scattered granulocytes were present in the lesions.

Treatment for about 24 hr. did not sterilize the lesions, but it brought about a reduction in the number of viable cocci. The lesions and the oedema fluid in 4 rabbits, 2 treated with penicillin and 2 with terramycin, were sterile after 34 to 66 hr. of treatment.

Microscopically, treatment prevented the development of the enormous numbers of cocci seen in normal and leucopenic untreated animals at death, although after 24 hr. many normal pneumococci remained as well as numerous Gram-negative forms of different sizes. No obvious morphological changes were seen in the cocci from the animals receiving penicillin, although many of the organisms were more deeply stained than normal. In the terramycin-treated animals the Gram-positive cocci were pleomorphic and swollen forms were frequently seen. In the sterile lesions the Gram-negative forms had dispersed into fine granular debris, although one or two normal looking Gram-positive cocci sometimes persisted.

A few granulocytes were scattered through the skin, but they were not aggregated into foci and showed no phagocytosis.

The macrophages in the sterile lesions showed no tendency to invade the areas containing bacterial debris, but were seen mainly near blood-vessels and just under the epidermis. They were never grouped together and the majority showed no phagocytosis. Rare macrophages, however, were filled with Gram-positive pleomorphic coccal debris.

DISCUSSION.

Normal rabbits and rabbits made leucopenic by repeated injections of benzene were infected intradermally with virulent Type 1 pneumococcus and treated systemically with either penicillin or terramycin. The lesions in the normal rabbits became sterile after treatment for 48 hours, and those in the leucopenic rabbits after treatment for 34 to 66 hours. In neither normal nor leucopenic rabbits were the lesions sterile after 24 hours' treatment. It is possible that under certain conditions granulocytes can assist antibiotics to eliminate virulent organisms, but in the present experiments, using large doses of penicillin and terramycin, such an effect was not observed. There was no histological evidence of more phagocytosis in the normal treated animals that recovered than in the untreated animals that died, nor was there a marked difference in the duration of treatment necessary to sterilize the lesions in leucopenic as compared with normal rabbits.

Very complete descriptions of the histological features of the pneumococcal skin lesion in untreated, normal and leucopenic rabbits have been given by Goodner (1928*a*, *b*, 1931) and by Rich and McKee (1934). A single observation

by Rich and McKee was not confirmed in the present experiments. These authors stated that " there is practically no phagocytosis of the bacteria (pneumococci) by the abundant leucocytes " in the skin of the non-immune animal. In the present experiments, using a fully virulent pneumococcus, there was certainly very little phagocytosis in the oedematous areas, but granulocytes containing pneumococci were frequently seen in the more compact parts of the lesion. The " surface phagocytosis " phenomenon described by Wood, Smith and Watson (1946) may account for the ingestion of these cocci. It is well known that in the peritoneal cavity of mice infected with virulent pneumococci, where the cells and the cocci float freely in the exudate, no phagocytosis by granulocytes occurs.

The pneumococci in the leucopenic animals treated with penicillin or terramycin became Gram-negative and dispersed into a fine granular debris. It is concluded that the organisms were killed by the antibiotics and then underwent autolysis, a mechanism described by Todd (1945) for the action of penicillin on pneumococci *in vitro*. The same sequence probably occurred in the normal treated animals, but it was not possible to identify Gram-negative bacterial debris in areas of intense inflammation.

The possibility that antibodies may have assisted in eliminating the infection in the leucopenic rabbits can be dismissed, since the ability of animals poisoned with benzene to produce antibodies is grossly impaired (Hamilton, 1931), and Rich and McKee (1934) showed that antibody specific for the pneumococcus afforded no protection in the absence of granulocytes.

The part played by macrophages remains to be considered. There were always a number of macrophages in the skin of the leucopenic animals. These cells were never aggregated into foci, and the majority showed no phagocytosis. The few macrophages that did show phagocytosis were often filled with pleomorphic Gram-positive cocci, but the number of such cells was so small, and the amount of extracellular bacterial debris so large, that their contribution to the clearing of the tissues must have been very small. On the other hand, in the sterile lesions of normal rabbits a great many macrophages containing Gram-positive material were seen among the fragmenting granulocytes. Much of the intracellular material was recognisable as the remains of pneumococci; the cocci appeared to be undergoing digestion, for they were of all sizes down to quite small granules. Thus in the normal animal there may be two mechanisms for the disposal of killed pneumococci, extracellular autolysis and intracellular digestion by macrophages.

It is concluded from these experiments that large doses of penicillin and terramycin, administered systemically, can exert a completely bactericidal action *in vivo*. They will sterilize the lesions in an otherwise fatal pneumococcal infection in rabbits without the aid of the cellular and humoral defences of the animal.

SUMMARY.

Normal rabbits and rabbits made profoundly leucopenic by injections of benzene were infected intradermally with virulent Type 1 pneumococcus and treated systemically with large doses of either penicillin or terramycin. The skin lesions in the normal and the leucopenic rabbits were sterilized in a similar period of time.

Histologically there was no more phagocytosis by granulocytes in the skin of the normal treated animals that recovered than in the untreated animals that died, but some phagocytosis of virulent pneumococci by granulocytes did occur in the lesions of the normal untreated rabbits.

The cocci in the skin of the leucopenic rabbits became Gram-negative under treatment with penicillin or terramycin, and were seen in the sterile lesions as masses of fine granules. The organisms were presumably killed by the antibiotic and then underwent autolysis.

The possible contribution of macrophages and immune bodies to the elimination of the pneumococci in the leucopenic rabbits is considered. It is concluded that penicillin and terramycin can exert a completely bactericidal action *in vivo* in the absence of the cellular and humoral defences of the animal.

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REFERENCES.

BIGGER, J. W.—(1944) *Lancet*, ii, 497.

EAGLE, H., FLEISCHMAN, R., AND MUSSELMAN, A. D.—(1950) *Ann. intern. Med.*, **33**, 544.

FLOREY, H. W., AND JENNINGS, M. A., in FLOREY, H. W., CHAIN, E., HEATLEY, N. G., JENNINGS, M. A., SANDERS, A. G., ABRAHAM, E. P., AND FLOREY, M. E.—(1949) 'Antibiotics.' Oxford (University Press), p. 337.

GOODNER, K.—(1928*a*) *J. exp. Med.*, **48**, 1.—(1928*b*) *Ibid.*, **48**, 413.—(1931) *Ibid.*, **54**, 847.

HAMILTON, A.—(1931) *Arch. Path.*, **11**, 434, 601.

HARRISON, P. E.—(1946) *J. infect. Dis.*; **79**, 101.

HEATLEY, N. G., GOWANS, J. L., FLOREY, H. W., AND SANDERS, A. G.—(1952) *Brit. J. exp. Path.*, **33**, 105.

HOBBY, G. L., LENERT, T. F., PIKULA, D., KISELUK, M., AND HUDDERS, M. E.—(1950) *Ann. N.Y. Acad. Sci.*, **53**, 266.

Idem, MEYER, K., AND CHAFFEE, E.—(1942) *Proc. Soc. exp. Biol., N.Y.*, **50**, 281.

JENNINGS, M. A., AND FLOREY, H. W., in FLOREY *et al.*—(1949) 'Antibiotics.' Oxford (University Press), p. 1186.

LEVADITI, C.—(1945) 'La Pénicilline et ses Applications Thérapeutiques.' Paris (Masson et Cie).

RANTZ, L. A., BOISVERT, P. J., AND SPINK, W. W.—(1946) *Science*, **103**, 352.

RICH, A. R., AND MCKEE, C. M.—(1934) *Johns Hopk. Hosp. Bull.*, **54**, 277.

SELLING, L.—(1916) *Johns Hopk. Hosp. Rep.*, **17**, 83.

SKINSNES, O. K.—(1948) *J. infect. Dis.*, **83**, 101.

SPICER, S.—(1950) *J. Lab. clin. Med.*, **36**, 183.

Idem AND BLITZ, D.—(1948) *Ibid.*, **33**, 417.

TODD, E. W.—(1945) *Lancet*, ii, 172.

WOOD, W. B., SMITH, M. R., AND WATSON, B.—(1946) *J. exp. Med.*, **84**, 387.

OBSERVATIONS ON THE VIRUS OF MOLLUSCUM CONTAGIOSUM.

J. C. MITCHELL.

From the Department of Bacteriology, London Hospital Medical College, London, E.1.

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THE virus of molluscum contagiosum has not been shown to be related to any of the known viruses, but some features suggest a possible relationship with viruses of the pox group.

The elementary body shares with pox viruses a size of approximately 300 mμ. (Boswell, 1947), and, as shown by electron microscopy (Rake and Blank, 1950), an oblong or brick shape, and it can be readily seen with the light microscope when stained by heavy mordanting methods such as those of Gutstein and Paschen, or that described by Weiss (1928) for staining bacterial flagella.

The inclusion body produced in human epidermal cells by molluscum virus is intracytoplasmic and, when mature, acidophilic, staining a brilliant red with eosin or phloxine ; these features are also shown by the inclusion bodies of cowpox, vaccinia and fowlpox. The lesions of molluscum contagiosum are found only in stratified squamous epithelium and the virus is therefore strictly epidermotropic : the lesions caused by viruses of the pox group are principally epidermal.

The lesions of molluscum contagiosum, once established in human skin, may persist for months or years : histological examination of the lesion shows a great degree of hyperplasia of prickle cells but little or no inflammatory response : the chronicity of the disease and the microscopical features of epidermal hyperplasia with prominent intracytoplasmic eosinophilic inclusion bodies call to mind the lesion produced by fowlpox in the skin of the fowl.

Investigation of a relationship between molluscum virus and other viruses is difficult, because attempts to transmit the virus to experimental animals have failed. Monkeys, apes, sheep, chickens, rabbits, guinea-pigs and mice have been used without success (Rivers, 1948 ; MacCallum, 1950).

Previous attempts to demonstrate antibody formation to molluscum virus have been unsuccessful. Van Rooyen (1948) attempted to agglutinate the elementary bodies with sera from human patients but failed to demonstrate the presence of specific agglutinins, and Brain (1937) tried treating the disease with vaccines made from molluscum lesions but without benefit.

It was decided to re-investigate this problem by testing patients' sera for the presence of complement-fixing antibody and, if necessary, inducing antibody formation by administration of a molluscum vaccine.

Attempts were made to transmit the infection to other hosts, but it was thought that even if reliance had to be placed on human lesions as the only source of antigen, a possible serological relationship with the pox group might still be demonstrable.

MATERIALS AND METHODS.

Sera.—Sera collected from patients infected with molluscum contagiosum were stored at 4° with the addition of sodium azide in a final concentration of 0·3 per cent. In each case the diagnosis was confirmed by histological examination of the lesion.

Antisera made in the rabbit against the viruses of vaccinia and cowpox were available in the laboratory.

Diluting fluid.—Egg material for passage was harvested into 0·02 M phosphate buffer at pH 7·4, and, for use as antigen in the complement-fixation test, was suspended in normal saline.

Antigens.—Molluscum nodules curetted from the skin of patients were weighed fresh and ground in a TenBroeck tube with the addition of sufficient sterile normal saline to make a 1 per cent w/v suspension. The suspension was clarified by centrifugation in a horizontal centrifuge for 10 min. at 2000 r.p.m. and the supernatant was removed and stored at 4°. In each case the supernatant was found to be infected with staphylococci, and was made sterile by the addition of sodium azide in a final concentration of 0·3 per cent.

Control antigens.—Normal human abdominal skin was obtained and as much corium as possible dissected away. This epidermis was weighed, ground and treated as the molluscum nodules and a 5 per cent w/v suspension was prepared. As it might be argued that this suspension, being in part fragmented corium, was insufficient as a control, curetted human verrucae vulgares were obtained and treated in the same way to prepare a 5 per cent w/v suspension.

As an additional control antigen the contaminating staphylococcus was grown in digest broth, deposited by centrifugation and re-suspended in sufficient normal saline to make a turbid suspension.

Vaccinia.—Vaccinia virus obtained from calf lymph was inoculated on to the chorio-allantoic membrane of the embryonated hen's egg. After 3 days' incubation infected membranes from the third passage were harvested into normal saline, 2 ml. per membrane, and ground in a TenBroeck tube. The suspension was centrifuged in a horizontal centrifuge for 10 min. at 2000 r.p.m. and the supernatant was removed for use as antigen.

Cowpox.—Cowpox virus which had been isolated from a human lesion and egg-adapted was treated in the same way as vaccinia.

Fowlpox.—A strain of fowlpox virus, kindly supplied by Mr. N. Dobson, Veterinary Laboratory, Weybridge, was used to infect chorio-allantoic membranes which, after 5 days' incubation, were treated in the same way as vaccinia.

Complement-fixation technique.

Sera were inactivated at 56° and a unit volume of 0·1 ml. of suitably diluted serum was used for each tube. The same volume containing 2 M.H.D. guinea-pig complement from a batch of pooled serum stored on dry ice was added and followed by 0·1 ml. of antigen in a suitable dilution. The usual controls were included and to all tubes 0·5 ml. of saline was added. The racks were then left at 4° overnight. Next day the racks were allowed to stand at room temperature for ½ hr., when 0·2 ml. of a 5 per cent suspension of sheep red cells sensitized with 5 M.H.D. of haemolytic amboceptor was added to each tube. The racks were placed in the water-bath at 37° for 30 min. when a first reading was taken, a second one being made after the tubes had stood overnight at room temperature. The highest dilution of serum giving complete fixation was taken as the end-point.

RESULTS.

Eleven of the 14 sera obtained from infected human patients gave no fixation of complement with the molluscum antigen. Three sera gave fixation, one with a titre of 1/8, two with titres of 1/4, but giving partial fixation at 1/8. The controls gave no fixation.

The distribution of lesions varied: in most cases they were mainly on the trunk and proximal parts of the limbs, but a child showed an infection limited to the face and an adult had two lesions only on one foot. The number of lesions varied from 2 to about 60. It was noted as of possible significance that the 3 cases possessing circulating antibody were those who had a heavy infection of the genital region; the patient with the heaviest infection, showing about 60 lesions on the upper trunk, possessed no circulating antibody.

It was decided to stimulate antibody formation in one of the eleven infected patients who showed no naturally occurring antibody: the patient selected was

aged 14 and had a history of infection for 6 months. The nodules were curetted from his skin, ground up in normal saline and the suspension was clarified by horizontal centrifugation for 10 min. at 2000 r.p.m. The supernatant, when removed, was found to be infected with a coagulase-negative staphylococcus, and formaldehyde was added in a final concentration of 0·2 per cent. After standing at 4° for 48 hr. the supernatant was found to be sterile culturally, both aerobically and anaerobically.

A course of 4 subcutaneous injections of this preparation was given, 0·5 ml. as a first dose and thereafter 1 ml. at approximately weekly intervals. No untoward local or general reaction occurred.

Clearly (Table I) the injection of the molluscum vaccine has induced the production of specific antibody: suitable controls, not shown in the Table,

TABLE I.—*Effect of Immunization with Molluscum Vaccine. Patient, ♂, aet. 14.*

Day.	Dose of molluscum vaccine.	Complement-fixation titre of serum.		
		1/4.	1/8.	1/16.
1	0·5 ml.	—	—	—
7	1·0 ml.	..	..	..
21	..	++++	—	—
36	1·0 ml.	+++	—	—
41	1·0 ml.	..	..	..
46	..	++++	++++	—

confirmed the specific nature of the fixation with molluscum antigen. As the patient's molluscum nodules had been removed before giving the immunizing injections, it was not possible to say whether they had any therapeutic effect. Serum from this immunized patient and the serum from a case of molluscum contagiosum with naturally occurring antibody (titre of 1/8) were now used in cross complement-fixation tests with vaccinia, cowpox and fowlpox antigens. The results given in Tables II and III show a complete absence of cross reaction between the virus of molluscum contagiosum and vaccinia or cowpox. In similar experiments it was found that fowlpox antigen failed to react with molluscum antisera.

The Nature of the Complement-Fixing Antigen of Molluscum Virus.

Since all attempts to transmit infection with molluscum virus to animals or the embryonated egg had failed, only two methods for the detection of the virus were available: microscopy of suitably stained smears and the complement-fixation test. It was important to find out whether the antigenic component fixing complement was present in the virus particles or whether it occurred free from them. A suspension of molluscum virus was centrifuged for 3 hr. in an angle centrifuge at 9000 r.p.m., the supernatant fluid was pipetted off and kept, and the deposit was made up to the original volume with saline. Smears were made from both fractions and stained by Weiss' method; the virus particles were present in the deposit, but were either absent or present in very restricted numbers in the supernatant. Complement-fixation tests were negative with the deposit, but as strongly positive with the supernatant fluid as with the original suspension. This finding was reproducible. The complement-fixing antigen of molluscum virus belongs to the so-called soluble antigens. It was found that

TABLE II.—*Cross Complement-Fixation Test between Molluscum and Vaccinia Viruses.*

Serum.	Dilution.	Antigens + 2 M.H.D.							
		Molluscum.			Vaccinia.			Normal skin	Normal C.A.M.
		1/8.	1/16.	1/32.	1/4.	1/8.	1/16.	1/4.	1/4.
Molluscum	1/4	++++	++++	+++	—	..	..	—	—
	1/8	++++	+++	+	..	..	..	..	..
	1/16	+	—	—	..	..	..	..	..
Vaccinia	1/8	—	..	..	++++	++++	++	—	—
	1/16	..	..	..	++++	++++	+	..	..
	1/32	..	..	..	+	—	—	..	..
Norman human	1/4	—	..	..	—	..	..	—	—
Normal rabbit	1/8	..	..	..	—	..	..	—	—

TABLE III.—*Cross Complement-Fixation Test between Molluscum and Cowpox.*

Serum.	Dilution.	Antigens + 2 M.H.D.							
		Molluscum.			Cowpox.			Normal skin	Normal C.A.M.
		1/8.	1/16.	1/32.	1/16.	1/32.	1/64.	1/4.	1/4.
Molluscum	1/4	++++	++++	+++	—	..	..	—	—
	1/8	++++	+++	+	..	..	..	..	..
	1/16	+	—	—	..	..	..	..	..
Cowpox	1/16	—	..	..	++++	++++	+	—	—
	1/32	..	..	..	++++	++++	—	..	..
	1/64	..	..	..	++	+	—	..	..
Norman human	1/4	—	..	..	—	..	..	—	—
Normal rabbit	1/8	—	..	..	—	..	..	—	—

the fixing power of this antigen was destroyed by heating for 1 hr. at 56°, and that it gradually disappeared when stored at 4° ; in 6 weeks it was much diminished or absent. Addition of sodium azide did not affect the activity of the antigen.

Attempts at Transmission of Molluscum Virus.

The attempts which were made to establish molluscum virus in the embryonated hen's egg are summarized in Table IV.

TABLE IV.—*Embryonated Egg Passage.*

Route of inoculation.	Number of eggs.	Age.	Inoculum.	Number of passages.
C.A.M.	5	7–10	Molluscum virus	2
Yolk-sac	6	3	,,	2
Allantoic cavity	8	10–13	,,	1
Amniotic cavity	23	7–9	,,	6
Intra-ocular	7	12	,,	2
Normal human skin grafted on C.A.M.	32	..	,,	2
Molluscum skin nodules grafted on C.A.M.	8	..	..	1
C.A.M.	10	9–14	Molluscum virus and boiled cowpox virus	2
,,	5	8	Molluscum virus and living vaccinia virus	6
,,	5	12	Molluscum virus and living fowlpox virus	3

Selbie (1946) reported the successful passage of Shope papilloma virus to the domestic rabbit when it was inoculated, together with living virus of contagious pustular dermatitis of sheep. Molluscum virus was inoculated on to chorio-allantoic membranes with heat-killed cowpox virus or with living vaccinia or fowlpox viruses, in the hope that molluscum virus might multiply with the aid of some factor present in these pox viruses. Eggs were examined at various stages for the development of lesions and suggestive histological changes.

In the experiments using heat-killed cowpox virus no macroscopic or microscopic lesions suggestive of virus multiplication appeared. Inoculation with living vaccinia or fowlpox viruses produced lesions characteristic of infection with these two viruses, and histological study showed the usual microscopical features ; after several passages infected membranes were used as antigen in complement-fixation tests with molluscum antiserum, with a negative result.

Following two 16-day passages in the yolk-sac no microscopic evidence of virus multiplication was found in stained sections of yolk-sac and embryo liver, and the presence of molluscum antigen could not be demonstrated by complement-fixation tests.

Molluscum virus was inoculated into the amniotic cavity. After 6 passages at 7-day intervals, using ground whole embryo and membranes as passage material, no microscopic evidence of virus multiplication was found : such material was found to be too anti-complementary for satisfactory use in complement-fixation tests.

Intra-ocular inoculation of molluscum virus with local and systemic cortisone produced no suggestive lesions.

Goodpasture and Anderson (1942, 1944) succeeded in transmitting zoster virus to human skin grafted on the chorio-allantois. In the experiments summarized in Table IV most of the human skin grafts inoculated with molluscum

suspension survived for 7 days, but in only 4 out of 32 was transfer of the graft to a second egg successful, and no inclusion bodies which could be confidently stated to be caused by molluscum virus were seen in stained sections. By grafting whole molluscum human skin nodules to the chorio-allantois it was hoped that molluscum virus, while still present in living human cells, would gradually adapt itself to chick ectoderm.

No unequivocal evidence of molluscum virus multiplication was obtained from any of these egg passages. Finally, attempts at transmission to suckling mice by intracerebral and intraperitoneal injections were also unsuccessful.

DISCUSSION.

It appears that infection with molluscum virus excites antibody formation in only a small proportion of cases. This is not surprising in view of the superficial nature of the infection. It is likely that in a larger proportion of patients absorption of small amounts of soluble antigen causes the establishment of the antibody-forming mechanism; the amount of circulating complement-fixing antibody is, however, too small to be detectable. Incidents which cause vascular congestion, such as irritation or bacterial infection of a lesion, would cause absorption of larger amounts of antigen and the production of detectable antibody. Formation of antibody, as a secondary response, would then cause the rapid disappearance of lesions, and this is commonly observed clinically as a sequel to secondary infection.

SUMMARY.

Three out of 14 patients with molluscum contagiosum possessed complement-fixing antibody to an antigen prepared from human lesions.

Antibody formation was induced in one patient by subcutaneous injection of molluscum virus.

The complement-fixing antigen of molluscum virus is soluble and heat-labile.

No serological relationship between molluscum virus and vaccinia, cowpox or fowlpox viruses could be demonstrated by the complement-fixation test.

Attempts to establish the virus in embryonated hens' eggs and in suckling mice were unsuccessful.

I have pleasure in thanking Professor S. P. Bedson and Professor C. F. Barwell for much help and encouragement, and Mr. L. W. J. Bishop for technical advice and assistance.

REFERENCES.

Boswell, F. W.—(1947) *Brit. J. exp. Path.*, **28**, 253.
Brain, R. T.—(1937) *Brit. med. J.*, ii, 1064.
Goodpasture, E. W., and Anderson, K.—(1942) *Amer. J. Path.*, **18**, 563.—(1944) *Ibid.*, **20**, 447.
MacCallum, F. O.—(1950) In 'Virus and Rickettsial Diseases.' London (Arnold), p. 189.
Rake, G., and Blank, H.—(1950) *J. invest. Derm.*, **15**, 88.
Rivers, T.—(1948) 'Viral and Rickettsial Infections of Man.' Philadelphia (Lippincott), p. 545.
Selbie, F. R.—(1946) *Brit. J. exp. Path.*, **27**, 143.
Van Rooyen, C. E.—(1948) 'Virus Diseases of Man.' New York (Nelson), p. 146.
Weiss, E.—(1928) *J. infect. Dis.*, **43**, 228.

PRECIPITIN REACTIONS IN THE PRESENCE OF MONO- AND POLYHYDRIC ALCOHOLS.

R. A. GRANT.

From the Department of Chemical Pathology, London Hospital Medical College, London, E.1.

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It has been known for some time that sugars tend to reduce the amount of precipitate formed in an antigen-antibody reaction (Landsteiner and Welecki, 1911; Downs and Goodner, 1926). It has also been shown that in the antigen excess zone where normally precipitation is reduced or inhibited, the presence of ethanol increases the amount of precipitate (Grabar and Oudin, 1945). In view of these findings, it seemed of interest to compare the rates of precipitin reactions in the presence of varying concentrations of simple mono- and polyhydric alcohols. A convenient method of following such reactions is to measure the changes of turbidity which occur when a solution of antigen is added to the antiserum (Pope and Healey, 1938). In general the turbidity rises sharply when the components are mixed, passes through a maximum and falls as the particles flocculate and settle out.

METHODS AND MATERIALS.

Turbidities were measured in a Hilger "Uvispek" spectrophotometer at a wave-length of 550 mμ., using aperture "A" and a slit width of 0·1 mm. The balancing cell contained a solution of antiserum of the same concentration as that in the reaction cell.

Four precipitin reactions were studied, crystalline horse serum albumin and rabbit antibody (Fig. 1), crystalline ovalbumin and rabbit antiserum (Fig. 2), pneumococcal polysaccharide Type I and horse antiserum (Fig. 3), and pneumococcal polysaccharide Type III and rabbit antiserum (Fig. 4). The horse serum albumin had been prepared by Mr. R. G. S. Johns using ammonium sulphate crystallisation at pH 4·9; the α_1-globulin was removed by passing through a column of powdered silica. The Type I polysaccharide and the crystalline ovalbumin were prepared by Mr. R. G. S. Johns by the methods of Heidelberger, Kendall and Scherp (1936) and Hopkins (1899) respectively. The rabbit antisera to ovalbumin and horse serum albumin were made by Professor J. R. Marrack by the method of Proom (1943). The horse antiserum to the Type I polysaccharide was made at the Lister Institute in 1939; it had been stored in the cold and contained a little phenol. The Type III polysaccharide (Lederle, lot 17) and the rabbit antiserum were provided by Dr. R. G. White. In the rabbit antiserum to horse serum albumin, the globulin fraction was precipitated by half saturated ammonium sulphate; it was re-dissolved and dialysed against buffered saline

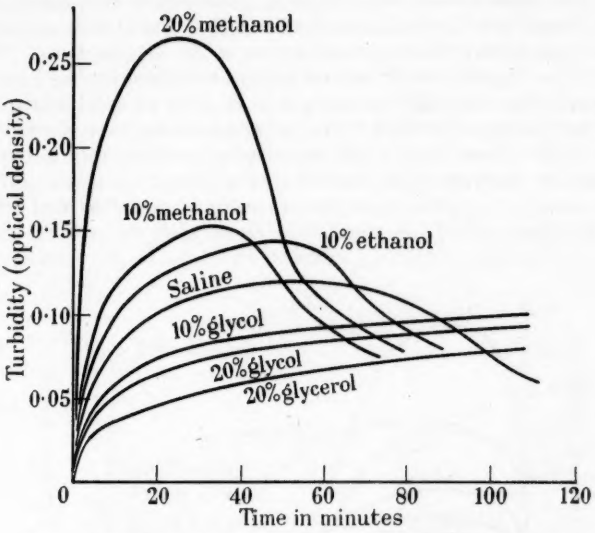

FIG. 1.—Reaction of horse serum albumin and rabbit antibody.

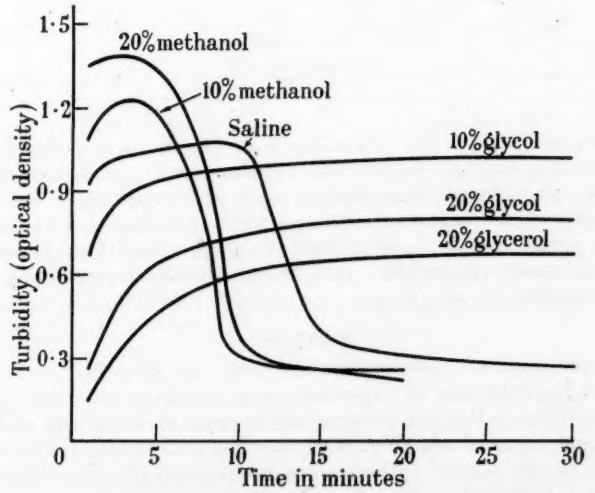

FIG. 2.—Reaction between ovalbumin and rabbit antiserum.

(pH 6·9) till free from ammonium sulphate ; antisera were diluted with buffered saline (0·15 M NaCl, 0·01 M phosphate buffer, pH 6·9) and well centrifuged before use. All the experiments were carried out at room temperature. The reactions between the Type I polysaccharide and the horse antiserum and ovalbumin and rabbit antiserum were carried out using a final antiserum dilution of 1:10, 2 cm. cells and a final volume of 5·0 ml. The other reactions were carried out in 1 cm. cells with a final volume of 2·0 ml. and the same final dilution of 1:10. The diluted antiserum was placed in a small pyrex centrifuge tube, and the amount of alcohol to give the required final concentration added, followed by the antigen solution. The contents of the tube were thoroughly mixed by inversion, and

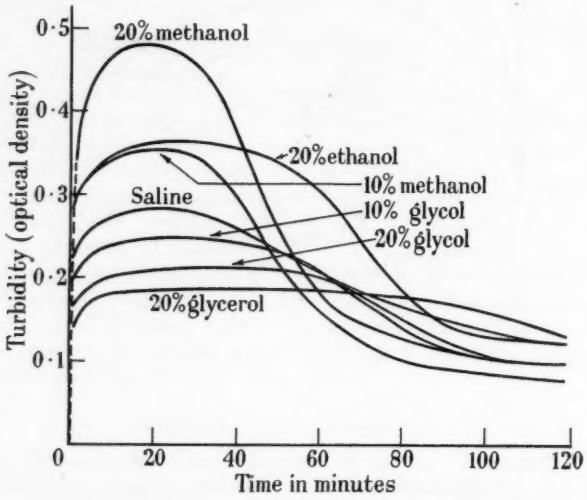

FIG. 3.—Reaction of pneumococcal polysaccharide Type 1 and horse antiserum.

transferred to the reaction cell. Readings were begun 1 min. after mixing, taken at 1 min. intervals for the first 10 min. ; subsequently at 5 min. intervals and again at 1 min. when the solution showed signs of flocculating.

The antibody contents of the sera used were as follows : horse anti-pneumococcus Type I 4·5 mg./ml., rabbit anti-pneumococcus Type III 4·5 mg./ml., rabbit anti-ovalbumin 4·6 mg./ml., and globulin solution containing antibody to horse serum albumin 3·1 mg./ml.

RESULTS.

To make the diagrams less cumbersome only the smoothed curves have been drawn and the large number of experimental points have been omitted.

The main effect of the monohydric alcohols on the reaction of horse serum albumin with rabbit antibody (Fig. 1) is greatly to accelerate the rate of increase of turbidity ; the maximum turbidity reached is also greater. The flocculation time (which can be taken as the point at which the turbidity starts to decrease)

is slightly reduced : this reduction is greater with methanol than with ethanol. The same general effects are found in the reaction of ovalbumin with rabbit antiserum (Fig. 2) ; here the effect on turbidity is rather less, and the effect on flocculation time somewhat greater than in the first reaction. Ethylene glycol and glycerol reduce the rate of increase of turbidity and greatly prolong the flocculation time ; in the presence of 20 per cent glycerol several hours may elapse before any visible precipitate forms at room temperature. The horse serum albumin—rabbit antibody reaction is more sensitive to these compounds than the corresponding ovalbumin reaction.

With the reaction of Type I pneumococcal polysaccharide and horse antiserum (Fig. 3) the presence of ethanol or methanol accelerates the turbidity rise and

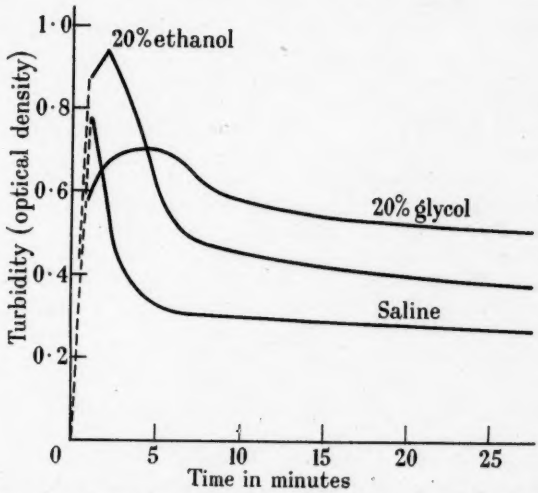

FIG. 4.—Reaction of Type III pneumococcal polysaccharide and rabbit antiserum.

the maximum reached. Ethanol slightly delays flocculation. Glycol and glycerol exert an inhibiting action but at a reduced level, and the effect on flocculation is much less than in the foregoing reactions. With Type III polysaccharide and rabbit antiserum (Fig. 4) ethanol increases the maximum turbidity reached ; flocculation is almost unaffected. Ethylene glycol decreases the maximum turbidity and delays flocculation, although these effects are not well marked.

DISCUSSION.

From the turbidity-time curves, it is seen that the rate of increase of turbidity is greater in the presence of monohydric alcohols, methanol being more effective than ethanol. Proteins are not precipitated by these concentrations of alcohol at room temperature. This effect is probably due to the decreased solubility of proteins in the presence of alcohol which lowers the dielectric constant of the

solution (Kirkwood and Scatchard, 1932). Hence, the antigen-antibody complexes would tend to precipitate more rapidly than from an aqueous solution.

In contrast, ethylene glycol has a marked inhibiting effect on the reactions, the rate of increase of turbidity being reduced and the flocculation time increased. Glycerol has a still greater inhibiting power. The introduction of a second or third OH group into the alcohol would increase its tendency to form hydrogen bonds. The combination of antigen with antibody is considered to depend mainly on Van der Waals interactions, ionic bonds and hydrogen bonds (Pauling, Campbell and Pressman, 1943). The strength of hydrogen bonds involving oxygen and nitrogen atoms is about 5 K cals. per mole. This is of the same order of strength as the attraction between isolated charges in water. The reactions which are most sensitive to the inhibiting actions of glycol and glycerol are those in which the antigens are natural proteins. The reactions involving the pneumococcal polysaccharide antigens are less affected, and of these the reaction between Type III polysaccharide and rabbit antiserum is less sensitive than the reaction between Type I polysaccharide and horse antiserum. The Type III polysaccharide is exceptional owing to the large number of carboxyl groups that it contains.

A reverse difference is found in the effect of high salt concentration; the precipitate formed by polysaccharide and antibody is dissociated (Heidelberger, Kendall and Teorell, 1936), while the precipitate from a natural protein and antibody is not (Marrack and Höllering, 1938). This supports the view that the reaction between a pneumococcal polysaccharide and antibody is ionic in nature. Additional evidence is provided by the fact that antisera to pneumococcus Types II and III cross-react with proteins coupled with glucuronic acid (Goebel, 1936; Woolf, Marrack and Downie, 1936). Cross reactions are found with antisera to pneumococcus Types III and VIII. Both of the polysaccharides contain an aldobionic acid group composed of glucose and glucuronic acid (Heidelberger, Kabat and Mayer, 1942). Antisera to Type II pneumococcus cross-react with plant gums containing uronic acids (Heidelberger, Avery and Goebel, 1929; Marrack and Carpenter, 1938).

Type III pneumococcus antisera give precipitates with artificial antigens (proteins) containing *p*-amino-carboxylic and sulphonic acids (Goebel and Hotchkiss, 1937). Also esterification of the uronic acid group of Type I polysaccharide abolishes the reaction with Type I antiserum (Chow and Goebel, 1935). There is no evidence that a similar ionizable group is an essential part of the determinants of natural protein antigens.

We would expect that the oppositely charged NH_3^+ groups of the antibody were essential parts of the receptors of pneumococcal antibodies but not necessarily of the receptors of antibodies to proteins.

Evidence for this based on the effects of formaldehyde is not very satisfactory, since formaldehyde appears to have other effects (*e.g.*, aggregation of protein molecules) on proteins besides combining with amino groups (Fraenkel-Conrat and Mecham, 1949). Eagle (1938) found that antipneumococcal sera to Types I and II when treated with formaldehyde could inhibit the formation of precipitates by untreated sera, although the treated sera themselves did not give precipitates with the polysaccharides. The flocculating ability of diphtheria antitoxin was also destroyed by formaldehyde treatment although combination with the toxin still took place.

We may infer that inhibition by high salt concentration is due to competition by cations for negatively charged groups in the polysaccharide. On the other hand, the inhibiting effects of ethylene glycol and glycerol may be due to the combination of these substances with hydrogen bonding sites on the antigen or antibody molecules. This would prevent the close approach of the specific combining surfaces of the molecules and also hinder the formation of hydrogen bonds between them. If this is so, then precipitin reactions with natural protein antigens would seem to be more dependent on hydrogen bonding than those with polysaccharide antigens.

The effects shown by glycol and glycerol are not found if a mixture of antigen or antibody with one of these substances is dialysed before the addition of fresh antibody or antigen respectively. Therefore no permanent changes have been produced in the proteins, and the combination of the glycol and glycerol with them must be reversible.

SUMMARY.

The effects of mono- and polyhydric alcohols on the rates of some precipitin reactions have been studied.

The rate of increase of turbidity is greater in the presence of ethanol and methanol.

Ethylene glycol and glycerol have an inhibiting effect ; this is greatest in those reactions where the antigen is a protein. It is suggested that the inhibiting effect is due to interference with the formation of hydrogen bonds.

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REFERENCES.

Chow, B. F., and Goebel, W. F.—(1935) *J. exp. Med.*, **62**, 179.
Downs, C. M., and Goodner, K.—(1926) *J. infect. Dis.*, **38**, 240.
Eagle, H.—(1938) *J. exp. Med.*, **67**, 195.
Fraenkel-Conrat, H., and Mecham, D. K.—(1949) *J. biol. Chem.*, **177**, 477.
Goebel, W. F.—(1936) *J. Bact.*, **31**, 66.
Idem and Hotchkiss, R. D.—(1937) *J. exp. Med.*, **66**, 191.
Grabar, P., and Oudin, J.—(1945) *Ann. Inst. Pasteur.*, **69**, 195.
Heidelberger, M., Avery, T., and Goebel, W. F.—(1929) *J. exp. Med.*, **49**, 847.
Idem, Kabat, E. A., and Mayer, M.—(1942) *Ibid.*, **75**, 35.
Idem, Kendall, F. E., and Scherp, H. W.—(1936) *Ibid.*, **64**, 559.
Idem, Kendall, F. E., and Teorell, T.—(1936) *Ibid.*, **63**, 819.
Hopkins, F. G.—(1899) *J. Physiol.*, **25**, 306.
Kirkwood, J. G., and Scatchard, G.—(1932) *Phys. Z.*, **33**, 297.
Landsteiner, K., and Welecki, S.—(1911) *Z. ImmunForsch.*, **61**, 464.
Marrack, J. R., and Carpenter, B. R.—(1938) *Brit. J. exp. Path.*, **19**, 53.
Idem and Höllering, H. F.—(1938) *Ibid.*, **19**, 424.
Pauling, L., Campbell, D. H., and Pressman, D.—(1943) *Physiol. Rev.*, **23**, 203.
Pope, C. G., and Healey, M.—(1938) *Brit. J. exp. Path.*, **19**, 397.
Proom, H.—(1943) *J. Path. Bact.*, **55**, 419.
Woolf R., Marrack, J. R., and Downie, A. W.—(1936) *J. Soc. chem. Ind., Lond.*, **55**, 156.

THE PURIFICATION OF ANTITOXIN BY ABSORPTION OF NON-ANTITOXIC ANTIBODIES.

C. G. POPE AND MURIEL F. STEVENS.

With technical assistance of J. C. MANUEL.

From the Wellcome Research Laboratories (Biological Division), Langley Court, Beckenham, Kent.

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IN work on the action of proteolytic enzymes on antitoxin it was shown (Pope, 1939*a*) that when normal horse serum was submitted to the action of pepsin there was a rapid digestion of more than 95 per cent of the initial protein. When the heat-denaturation process (Pope, 1939*b*) was applied to normal horse serum almost all the original protein was lost. On the basis of these results it would have been expected that the application of these methods would lead to a uniform purification of antitoxin regardless of the initial titre of the antitoxic serum employed, had the antitoxin been the only new pepsin-resistant protein produced as a result of hyperimmunization. The results, however, showed very clearly that this was not the case. Generally, horses that had immunized rapidly to high titre gave good results—that is, a product with a high value in terms of units of antitoxin per gramme of protein. The sera from horses that had responded poorly to immunization gave, as a rule, low " purity figures " in the peptic concentration process.

Some further light was thrown on this problem when Pope, Stevens, Caspary and Fenton (1951) reported that in addition to the specific antitoxin present in anti-diphtheria serum there was present also a series of antibodies to antigens present in *C. diphtheriae* culture filtrates, and moreover that these other antibodies behaved in the peptic process as did the antitoxin itself. Thus, the final product obtained after peptic concentration contained a series of antibodies in addition to the antitoxin of which only the antitoxin was determined quantitatively. Pope *et al.* have indicated that at least 24 antibodies were present in some of the antitoxic sera which they examined. The presence of a large number of antigens in the culture filtrates from *C. diphtheriae* may account for the failure of some horses to give a good antitoxin response on immunization, since it is only the antitoxin that is determined and not the total production of antibodies of different kinds. For example, a horse showing a poor antitoxin response may have produced large amounts of one or several other antibodies. If this is, in fact, the explanation for the low unit per gramme protein figures for certain antitoxic sera after peptic concentration, then it should be possible to show that almost all the protein that has survived the action of pepsin, followed by heat denaturation, has antibody properties of one kind or another.

The most direct way of showing this would be by the precipitation (flocculation) of the non-antitoxic antibodies with their antigens, leaving in this way

the specific antitoxin as the only remaining immunologically reactive protein. This, of course, requires the availability of all these antigens free from specific toxin, and it is in this respect that we have met our greatest difficulties in this work. However, considerable progress has been made, and it is the object of this paper to show that there is much evidence to support the view that the low unit/g. protein value obtained after peptic concentration of antitoxic sera from horses showing a poor response on hyperimmunization is due to the presence of other antibody proteins diluting the true antitoxin.

MATERIALS AND METHODS.

Antitoxin.

For most of the work reported here antitoxin concentrated by the peptic method (Pope, 1939*b*) was used; we selected a batch of material which had the very low purity figure of 16,000 units/g. protein. If it could be shown that the bulk of the protein present could be accounted for as antibodies other than antitoxin it would be clear proof of the view expressed previously.

In addition we have used antitoxic serum derived from a horse specially immunized with diphtheria toxoid which had been submitted to heat treatment. It was reported by Pope *et al.* (1951) that crude culture filtrates from *C. diphtheriae* contained a large number of proteolytic enzymes (about 17), and that these were heat-labile. When diphtheria toxoid was heated rapidly to a temperature of 90°, held at this temperature for a few seconds and then rapidly cooled, it was found that the proteolytic enzymes were inactivated and other antigens had their antigenic properties impaired. There was no loss of Lf in the *in vitro* test, indicating no loss of combining value with antitoxin, and no obvious change in the time of flocculation (Kf). However, the use of "flash-heated" toxoid for the hyper-immunization of the horse resulted in the production of serum giving far fewer lines in the diffusion test reported by Pope *et al.* (1951), indicating the production of a smaller number of antibodies than usual. This serum (MS 381) was studied both after concentration by ammonium sulphate (30–50 per cent saturation fraction) and peptic concentration (Pope, 1939*b*). Other batches of peptic antitoxin were used on a smaller scale in order to confirm certain points.

All the antitoxins used in this work were assayed by both *in vivo* and *in vitro* methods and the "Lr/Lf ratio" was substantially 1·0. This was also true for absorbed antitoxins which were tested similarly. Both tests were related to the International Standards through our laboratory sub-standards.

Antigen Sources.

Three types of products containing antigens have been used in this work:

(*a*) Culture filtrates from *C. diphtheriae* grown in the presence of excess iron to inhibit toxin production.
(*b*) Suspensions of *C. diphtheriae* in 50 per cent diethylene glycol.
(*c*) Culture filtrates from *C. diphtheriae* in which the specific toxin has been destroyed.

These are described more fully below.

Excess iron culture filtrates.

The effect of a high iron content in culture medium used for the production of diphtheria toxin was reported by Pope (1932), who showed that although growth of *C. diphtheriae* was quite good on iron-rich medium, the production of toxin was almost inhibited. Later, Pappenheimer and Johnson (1937), working with a medium of different composition, extended our knowledge of the rôle of iron in the production of toxin, and also showed that in the presence of 0·5 mg. iron/litre of medium, toxin production was practically inhibited. Culture filtrates from *C. diphtheriae* grown in the presence of excess iron (4·0 mg. $Fe^{\cdots}$/litre) using tryptic digest medium (Linggood and Fenton, 1947) have been employed in this work. The amount of iron required to inhibit toxin production depends on the composition of the medium used. These culture filtrates were concentrated by ultrafiltration using ultrafilters of the type described by Harms (1948). Based on the reduction in volume, a normal toxic

culture filtrate with an original value of about 80 Lf/ml. would have had a final value of about 4000 Lf/ml. The iron-filtrate concentrates had a value of 0–4 Lf/ml. (determined by blend test (Glenny and Okell, 1924)) on the concentrates. It is clear, therefore, that even after concentration these excess-iron culture filtrates are substantially free from specific toxin. It was hoped that these culture filtrates would provide a complete source of antigens, other than the specific toxin, but, as will be shown in this paper, this proved not to be true.

Antigens from C. diphtheriae.

We used the collected *C. diphtheriae* available from the large scale production of toxin or from cultures from excess-iron medium. The well-washed bacteria were pressed until fairly dry and then suspended to form a thick cream in 50 per cent diethylene glycol/water. Morgan (1937) used anhydrous diethylene glycol for the extraction of toxic O antigenic complexes from the smooth strains of *Sh. shigae*. The extraction of dried *C. diphtheriae* with anhydrous diethylene glycol appeared to be less effective than the use of 50 per cent diethylene glycol.

These suspensions were readily agglutinated by crude peptic antitoxins, and could be used for absorption of some of the antibodies present.

Other antigen preparations were obtained from these bacterial suspensions, for example, by digestion with trypsin or papain. The soluble products were tested for antigens or haptens by the diffusion method described by Pope *et al.* (1951) and by the optical method described later in this paper. Only a portion of the total antigen system was found in these preparations.

Antigens derived from C. diphtheriae *crude toxin filtrates.*

In the course of this work we made many attempts to find a method whereby the specific toxin could be destroyed without the destruction of the other antigens present in the crude toxic filtrate. We were not completely successful in achieving this, but through the use of pepsin at controlled pH values we were able to destroy almost all the toxin, and yet leave many of the antigens in a form where they flocculated readily with crude peptic antitoxin. There is, however, evidence that some of the antigens present resembled the specific toxin in that they too were destroyed by pepsin. For most work we found it preferable to use ultrafiltered toxin, diluted to 500 Lf/ml. and adjusted to pH 4·7–5·0, the digestion being carried out at 20° for several days. The amount of pepsin was not critical, and we used 0·5 g./litre of 1/3000 B.P. strength pepsin. Small samples, neutralized to pH 8–8·5, were tested at intervals using the blend test (Glenny and Okell, 1924) until the value of the specific toxin had fallen to less than 4 units/ml. This was less than 1 per cent of the original value. The blend test was carried out by adding to a toxin of known Lf value an equal volume of the neutralized sample and determining the Lf value of this mixture. The value found was equal to half the Lf of the test toxin plus half the value of the unknown sample in Lf u./ml. It may be pointed out here that the final product gave a direct flocculation at a value substantially the same as the original value of 500 Lf/ml. due to the presence of pepsin-resistant antigens. The flocculation time (Kf) is rather long unless the test is carried out with about 100 antitoxin u./ml. We refer to the product as " peptic-toxin " in this paper. The peptic-toxin does not neutralize the specific antitoxin except for the small trace of residual toxin shown by the blend test to be present. When the 500 Lf/ml. toxin was examined by the diffusion line method (see later) before and after peptic treatment, it was found that there was no marked change in the line structure. The major line, for example, was present in both tests, notwithstanding the fact that the specific toxin had been destroyed in the peptic-toxin product. It is clear that the specific toxin contributes only a small part to the series of reacting lines which go to make up this " major line," which Pope *et al.* have already shown is a compound system of different antigens and antibodies reacting very close to each other.

The destruction of toxin by means of acid alone was always incomplete ; even at pH 3 and after several days at 20°, combining power tests (*in vitro*) showed that 30–50 per cent of the specific toxin retained the power of combining with antitoxin. Trypsin acting at 20° and pH 7–8 behaved somewhat like pepsin in its action on toxin ; the specific toxin was destroyed fairly rapidly and trypsin-resistant antigens (haptens) remained. However, pepsin appeared to be the better enzyme for our purpose.

The enzyme papain, either activated with thioglycollic acid or un-activated, had little effect on toxin at a pH of 7–8 at 20° or 37°. In this pH range papain produced marked

proteolysis of crystalline horse haemoglobin. We do not know why the toxin should be stable to the action of papain and we are continuing this work at other pH values.

Methods for Determining the Purity of Antitoxins.

The diffusion method in gelatin.

Pope *et al.* (1951) have described their modification of the method of Oakley and Fulthorpe (1952) for the examination of diphtheria toxin and antitoxin. Bowen (1952) has reported his experiences with this method, but has introduced some modifications. For gelatin he has substituted agar gel, and worked at 37° in order to obtain quicker results. Our own experience is against this modification. Pope and Healey (1938) showed that toxin-antitoxin floccules were not re-dispersed in the cold by an excess of added antitoxin, but did so rapidly when warmed to 37°. This affects the definition where two fine lines appear very close to each other, and the fine line structure is more clearly defined when the tests are carried out in the cold. This is most important where two lines appear which are very close to each other. Moreover, Bowen has reduced the concentration of antitoxin and antigens used in the test.

We wish to stress here the importance of testing purified (absorbed) antitoxins against the crudest form of toxin available, and conversely of testing purified toxins against the most impure antitoxins available ; in all cases the strength of antigens and antibody should be as high as possible and preferably over 2000 u./ml. The length of the blank gelatin columns may with advantage be extended to 4·0 cm. when working with highly purified products although this increases the time required for the test, but it allows of the detection of very small amounts of antibody impurities which would be missed on the shorter column.

Optical methods.

The reaction between diphtheria toxin and antitoxin was studied quantitatively by Pappenheimer and Robinson (1937) who determined the nitrogen content of the washed "floccule precipitates." This is a standard procedure for the estimation of precipitates of this nature, but unfortunately very time-consuming when a large number of mixtures have to be examined, and it gives little indication in mixtures where the precipitation is either incomplete or the amount of precipitate is very small. Pope and Healey (1938) used optical density methods in a study of reactions between toxin and antitoxin ; Goldberg and Campbell (1951) studied changes in light scattering in the reaction between bovine serum albumin and purified rabbit antibody, and Gitlin and Edelhoch (1951) similarly studied the reaction between human serum albumin and the homologous horse antibody. These last workers found that the results obtained were independent of the method of mixing the reagents. Doty and Edsall (1951) consider that light-scattering methods have many advantages in the study of reactions between antigens and antibodies.

We have used a method which has been elaborated from the earlier method of Pope and Healey (1938). The optical instrument used for most of our work was built in the laboratory, and consists of a monochromator based on the principle of Hartridge (1915) using a diffraction grating and right-angle prism ; the transmitted monochromatic light falls on a selenium barrier-type photo-cell, the output of which is amplified by a Tinsley amplifier (Tinsley, 1941) to give readings in percentage transmission on a meter with a scale length of about 6 inches (15 cm.). It is not proposed to discuss this apparatus in more detail here, but to give instead the details of a simpler apparatus recently constructed and tested which depends on the same optical principles. This is shown in diagrammatic form in Fig. 1. The light source is a car head-lamp with a straight filament, fed from a constant voltage transformer ; the light passes through the pair of lenses to give an image of the filament falling on the entrance slit, with the filament image slightly greater than the dimensions of the slit. Mounted behind the slit are two Ilford "spectrum filters"—orange No. 607—and behind these is the barrier-type photo-cell, the output from which is fed to the 5000 ohm potentiometer across the galvanometer (Cambridge Instrument Co. Ltd. spot type resistance 450 ohm with a scale calibrated 0–100). The essential point for both these instruments is the position of the cell containing either the blank solution or the suspension whose density is being measured. It is placed in the cone of light emerging from the lens and falling on the entrance slit. In this position any divergence of the light due to scatter prevents it from falling on the entrance slit. The sensitivity is to some extent governed by the cone angle of light

falling on this slit ; a lens of short focal length tends to produce an instrument with a higher sensitivity (*i.e.*, the density reading is greater for a suspension of a given strength). Both types of instruments were tested using suspensions of different kinds, and each gave a linear relation between optical density (log 100–log per cent transmission) and concentration until the amount of transmitted light was less than 10 per cent of the incident light.

The method used for the examination of antitoxin (or toxin fractions) is as follows :

The antitoxin in any mixture was held constant at 20 u./ml. and the amount of toxin added was varied ; all mixtures were brought to constant volume with buffered saline (pH 7·8) containing 1/10,000 Thiomersalate (B.D.H.) as an antiseptic. Both antitoxin and toxin solutions used were diluted to contain as nearly as possible 100 u./ml. and the required volumes measured using grade A burettes. The mixtures were made in 25 ml. volumetric flasks, the toxin added first and then the antitoxin, after which the mixtures were made to volume. Each mixture, therefore, contained a constant amount of antitoxin, 500 units in 25 ml., which had been accurately determined against a test toxin, previously standardized against the sub-standard flocculation antitoxin. In many cases the antitoxin was also determined by *in vivo* tests and the two results usually agreed to about 3–4 per cent. The

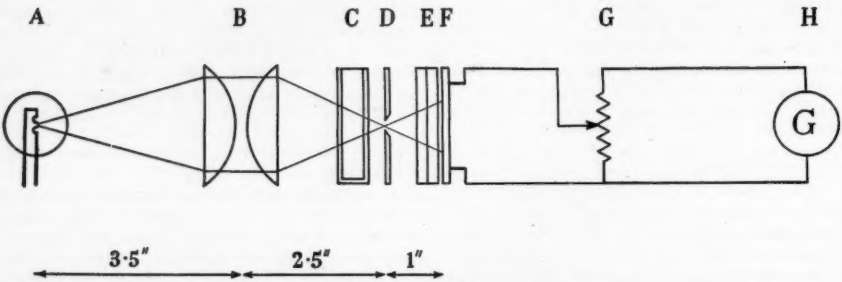

FIG. 1.—The optical method used for density determinations. A. Car headlamp bulb, 6 volts, 36 watts. B. Condenser lens, double plano-convex, 1·75 in. diameter. C. 1 cm. path optical cell, back of cell 0·375 in. from slit. D. Slit diaphragm 0·254 mm. × 1·0 cm. (max.). E. Two Ilford filters, No. 607, 2 in. square. F. "EEL" selenium photocell. G. Berco 5000 ohm wire-wound potentiometer, 4 watts. H. Cambridge spot galvanometer, scale graduated 0–100.

equivalence point for the toxin was determined by carrying out flocculation tests in which the toxin was varied and the antitoxin held constant. This is the reverse of normal practice in the flocculation method. For most samples of toxins and antitoxins the results agreed within the limits of experimental error independent of the method used. The volume of toxin giving the first flocculating mixture, *i.e.*, the volume equivalent to 500 units of the antitoxin, was taken as 100 per cent and mixtures containing from 20 to 240 per cent of this volume of toxin in 10 per cent steps were prepared. These mixtures were transferred to 50 ml. bottles and heated in a water-bath at 40°, usually for 1½ hr. They were then kept at room temperature until the next day before examination. Two optical readings were made on each mixture, one on the total mixture, and the other on the supernatant liquid after centrifuging at 2000 r.p.m. for 20 min. The difference between the optical density values for these two readings, *i.e.*, total density–supernatant density, gave the density value for the precipitated floccules. When working with colourless antitoxin solutions and purified toxins the use of water in the reference cell was sufficient, but with crude toxins which were markedly coloured suitable blank values were determined and corrections made.

Before reading the total densities of the mixtures each bottle was inverted at least 10 times, with care to avoid the production of bubbles, in order to re-disperse the floccules. After this treatment consistent density values were obtained over a period of some minutes ; only a few seconds are necessary to make an actual reading. The repeatability of the method has been checked on many occasions ; the results for one such test were : 5 separate mixtures,

each at 100 per cent toxin, were prepared and these showed optical density values of 0·319, 0·324, 0·319, 0·324 and 0·324 (mean 0·322).

In our experience this method is a valuable one; in the course of this work we have prepared well over 300 curves, generally over the range 0–240 per cent toxin, but in some cases over a much wider range. This would have been almost impossible had it been necessary to rely on nitrogen determinations, which would have provided less information about the course of the reaction between the antigens and antibodies studied.

During the course of this work observations have been made, using *in vivo* tests, on the presence of excess toxin or antitoxin in (*a*) total mixtures and (*b*) supernatants; also on the antitoxin recoverable from floccules and supernatants after pepsin treatment. Our findings will form the subject of a later paper.

Nitrogen determinations.

All nitrogen determinations were carried out by a micro-Kjeldahl method employing copper and selenium as catalyst (selenium oxychloride ($SeOCl_2$) 0·2 per cent w/v, copper sulphate pertahydrate ($CuSO_4.5\,H_2O$) 0·5 per cent w/v in 50 per cent v/v H_2SO_4, A.R.). The distillation apparatus described by Markham (1942) was employed and titrations were done with a micrometer syringe (Trevan, 1926).

The protein content of the antitoxins was determined in the following way: a suitable dilution, *e.g.*, 1:5, was made with a buffer (5 per cent w/v sodium acetate adjusted to pH 4·9 with acetic acid) and the protein coagulated by boiling. This operation (in triplicate) was carried out in centrifuge tubes and the coagulated protein centrifuged down; the coagulum was well washed with buffer and its nitrogen content determined. The factor 6·25 was used to convert nitrogen values to protein values.

RESULTS.

The reaction of the original antitoxin with toxin.

The original peptic antitoxin DP 1252 was first examined by preparing a series of mixtures with crude toxin in which the antitoxin content was constant at 20 u./ml., and the amount of toxin varied from 20 to 240 per cent in 10 per cent steps as already described. In Fig. 2 the results for this experiment are shown as total optical density (A) and floccule density (B) for each mixture. Two points are readily seen from these results—that the curves are not regular in shape, and that precipitation was never complete in any of the mixtures, as shown by the difference in the two curves. The supernatants from the centrifuged mixtures showed considerable optical density. Pope and Healey (1939) drew attention to the fact that the supernatants from neutral toxin-antitoxin mixtures frequently showed marked optical density indicating incomplete precipitation of the floccule complex. We shall return to this point later when considering some of the absorbed antitoxins.

In examining the interaction between toxin and antitoxin it was considered necessary to make the maximum number of observations on mixtures with steps not greater than 10 per cent in the amount of toxin present. In some experiments we have reduced this to 5 per cent increments of added toxin.

The reaction of "iron-antigen"-absorbed antitoxin with toxin.

The absorption of the peptic antitoxin DP 1252 with the excess-iron culture filtrates (ultrafiltered), already described, was carried out in the following manner. The volume of excess-iron culture filtrate required to produce optimal flocculation with the antitoxin was determined and a bulk mixture in these proportions was

then prepared. We found it advisable to have the antitoxin at a value between 100 and 500 u./ml. and to add the iron-antigen in concentrated form. Under these conditions rapid flocculation took place and the final volume was not excessive. The floccules were separated on the centrifuge and washed twice with saline, the washings being added to the main supernatant liquid. Much colour was introduced into the antitoxin-containing supernatant from the iron-antigen, but most of it was removed by adjusting the pH to 3·5 and filtering off the precipitate formed. Notwithstanding the bulky precipitate of floccules produced by interaction of this antigen system and peptic antitoxin, little or none of the specific antitoxin was lost in this precipitate.

This antitoxin purified by absorption was tested by the diffusion method against both crude toxin and against the excess-iron concentrate with which it had been absorbed. The crude toxin produced, in addition to the major line, a number of other lines, whereas the excess-iron concentrate gave no reaction. Examination of this absorbed antitoxin by the optical method gave the result shown in Fig. 3. The crude test toxin was that used before (Fig. 2), and other conditions were the same. It will be seen that after absorption with the excess-iron antigen system, the standard amount of antitoxin now gave mixtures with less density indicating a reduction in the amount of floccule precipitate. The dotted line in Fig. 3 shows the maximum density given by the unabsorbed antitoxin. Here again the shape of the curve is irregular. The reason for these irregularities in the curve will be discussed later.

The reaction and absorption of antitoxin with "peptic-toxin."

The method used for the preparation of peptic-toxin has already been described, and we now consider its use for the absorption of some of the non-antitoxic antibodies in the crude peptic antitoxin. Experimental work showed that the absorption was best carried out in a fractional manner as follows: For ease of description we refer to the peptic-toxin as though it still contained its original value of 500 Lf u./ml., although in fact the specific toxin has been reduced to less than 1 per cent of its original value. Starting with a convenient amount of crude peptic antitoxin (*e.g.*, 500,000 units) we added a volume of peptic-toxin which, based on its *original value*, would have supplied 250,000 Lf units. The composition of this mixture is referred to as TA_2, indicating one toxin equivalent to two antitoxin equivalents. This mixture flocculated heavily and rapidly; it was centrifuged and the precipitate washed with saline, the washings being added to the main supernatant.

To the supernatant the same volume of peptic toxin was again added to produce a mixture of the composition TA. This produced a second somewhat smaller precipitate, which was centrifuged off and washed as before. The supernatant was again treated with the same volume of peptic-toxin to give $T_{1\cdot5}A$ mixture. In this way the additions of peptic-toxin were continued until no more floccule-precipitate was produced and the mixture had the final composition of T_4A. The last supernatant, corresponding to T_4A, contained about 80 per cent of the original antitoxin units as determined by both *in vivo* and *in vitro* tests. The loss was due to that resulting from specific combination with residual traces of toxin, together with mechanical loss in centrifuging and incomplete washing of the discarded floccule precipitates. After this absorption the antitoxin was

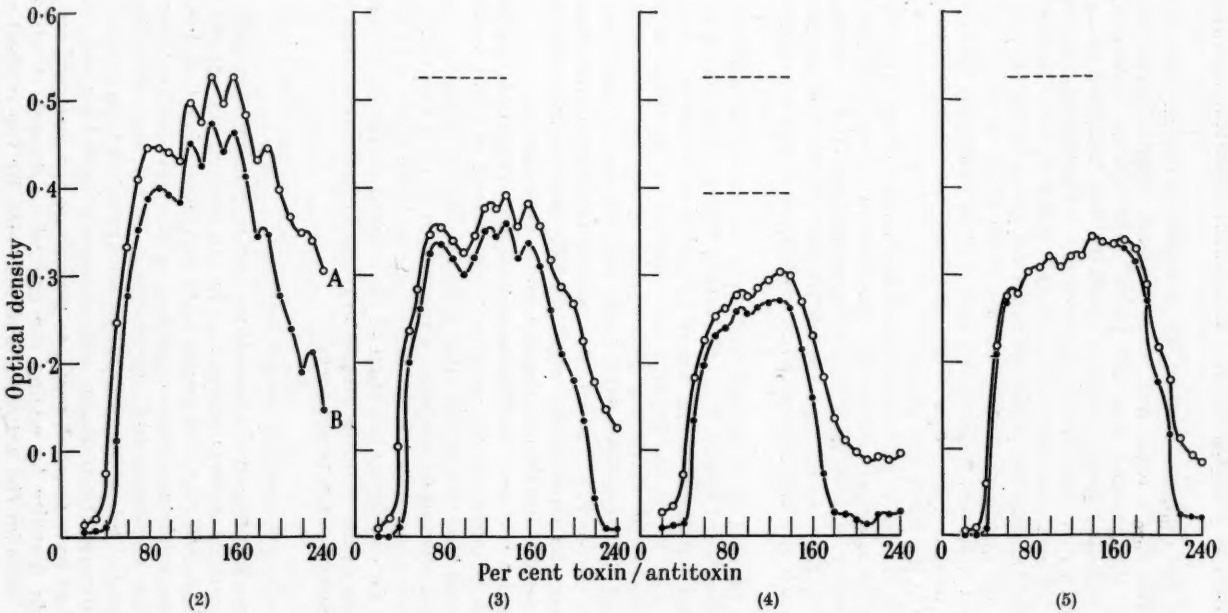

FIG. 2.—The reaction between the original peptic-antitoxin and crude toxin. Final antitoxin concentration 20 units/ml. in each mixture. The first flocculating mixture is at 100 per cent toxin. A ⊙ Total density. B ● Floccule density.

FIG. 3.—The reaction of the peptic-antitoxin with crude toxin after absorption with antigens present in excess-iron culture filtrate. Dotted line = maximum density from Fig. 2. Other data as in Fig. 2.

FIG. 4.—The reaction of T_4A antitoxin with crude toxin. Dotted lines, upper = maximal density from Fig. 2; lower = maximal density from Fig. 3. Other data as in Fig. 2.

FIG. 5.—The reaction between the original peptic-antitoxin and a toxin with a purity of 2560 Lf/mg. P.N. Dotted line = maximal density from Fig. 2. Other data as in Fig. 2.

recovered and concentrated by precipitation with ammonium sulphate in the usual way.

When examined by the diffusion method the absorbed antitoxin gave one broad major line against crude toxin and some reaction against excess-iron antigen. Quite clearly this product was not yet a single system containing antitoxin as the only immunologically reactive antibody, but the tests by the optical method indicated a very considerable improvement. The results obtained in the optical method between this absorbed antitoxin (called T_4A) at constant antitoxin value (20 u./ml.) against the crude test toxin are shown in Fig. 4. The two dotted lines show the maximum density for the crude peptic antitoxin and the excess-iron absorbed antitoxin respectively, and it is at once clear that the floccule density values produced by "T_4A antitoxin" have been much reduced. However, irregularities in the curve still persist.

It was thought to be of interest to compare the behaviour of a highly purified toxin instead of the crude material, and for this purpose we prepared mixtures using a toxin with a purity of 2560 Lf u./mg. protein nitrogen (P.N.), the test antitoxins used being (*a*) the original peptic antitoxin and (*b*) the T_4A material prepared from it. In each case the final antitoxin concentration was constant in all mixtures at 20 u./ml.

The results for the purified toxin against the peptic antitoxin are shown in Fig. 5, and for the purified toxin against T_4A antitoxin in Fig. 6. Comparing the results it will be seen that the curves in Fig. 5 show greater density values and are broader than those in Fig. 6. It has already been pointed out by Pope *et al.* (1951) that highly purified toxin contains a large number of antigens detectable in the diffusion test, and the reactivity of this toxin with the crude peptic antitoxin is not surprising. From Fig. 5 it will also be seen that in mixtures from 70 to 160 per cent toxin, the supernatants were optically clear, as indicated by the fact that the total density and floccule density values were identical.

Comparing Fig. 4 and 6 it is seen that flocculation is less extensive when the absorbed antitoxin T_4A is tested against purified toxin instead of crude toxin, and this in itself indicates that antibodies other than specific antitoxin are still present in T_4A, since the amount of antitoxin was constant in each experiment. It is clear that the peptic-toxin does not contain all the antigens, other than the specific toxin, required for complete absorption of the original antitoxin. There are indications that some of the antigens present in crude toxin are destroyed as easily as toxin by the action of the pepsin.

The absorption of antitoxin with combined antigen materials.

Since no single antigen source proved adequate we have carried out absorptions making use of all the antigen systems available. In this method the antitoxin may, for example, be absorbed first with peptic-toxin and then, after re-concentration with ammonium sulphate, further absorbed with excess-iron antigens. A final absorption with a suspension of *C. diphtheriae* in 50 per cent diethylene glycol is sometimes helpful. The addition of antigen sources of this type naturally leads to the contamination of the antitoxin with proteins provided by antigens present in excess, and we have found it useful to precipitate the absorbed antitoxin as toxin-antitoxin floccules by adding the equivalent amount of toxin. From the well-washed floccules the antitoxin was recovered by the method of Pope and Healey (1939), except that the minimal amount of crystalline pepsin

was used instead of crude pepsin. A recovery of 80 to 90 per cent of the antitoxin in the floccules has been obtained in this way.

Details of one purification carried out in this way are given here as an illustration of the method. It is admittedly very time-consuming, but we have found no short cuts which give equal results.

An attempt was made to isolate antitoxin with the closest approach to a homogeneous material that the methods already indicated allowed. The crude peptic antitoxin DP 1252 was first converted into floccules by the addition of an equivalent amount of crude toxin and the resulting floccules were well washed. Antitoxin was recovered from these floccules by the method of Pope and Healey (1939). It was obviously heavily contaminated with other antibodies, as shown by diffusion tests and the optical method. This " floccule antitoxin " was then fractionally absorbed with peptic-toxin through the stages TA_2, TA, $T_{1\cdot5}A$, T_2A, $T_{2\cdot5}A$, T_3A and T_4A, precipitates being removed at each stage. Further purification with excess-iron antigen was then used to flocculate other unwanted antigen-antibody systems. After this treatment the absorbed antitoxin (88 u./ml.) was re-concentrated with ammonium sulphate. It was re-precipitated as floccules using an equivalent amount of toxin and the antitoxin recovered (90 per cent yield) from the washed floccules (crystalline pepsin 0·0125 per cent, pH 3·0, 1 hour digestion at 20°). The final product obtained contained 1110 (*in vitro*) u./ml. and had a purity of 211,000 antitoxic units per g. protein. By the diffusion test this antitoxin appeared to be quite homogeneous ; it gave a single very narrow line against crude toxin and a series of toxin fractions against which it was tested. However, the more searching optical test showed that it was heterogeneous, and it is possible that the apparent single diffusion line was built up of a series of reacting systems. It is proposed to deal with the reactions of this isolated antitoxin fraction in detail in the next paper.

The absorption of special antitoxin MS 381.

Using flash-heated toxoid we obtained, as already described, an antitoxic serum in which the overall antibody production had been restricted. From this serum the antibody globulin was separated with ammonium sulphate between the limits 30 to 50 per cent saturation. The product examined by the optical method was obviously impure ; it was therefore absorbed with peptic-toxin and excess-iron antigens in the manner already described. After the removal of non-antitoxic antibodies, the absorbed antitoxin gave the result shown in Fig. 7 when tested against crude toxin. It will be seen that the curve is almost symmetrical, although this was not so before absorption. We have also purified a portion of this antitoxic serum by the peptic method, and made preliminary studies on the absorption of the antibodies, but this work is still incomplete.*

It is possible to produce an absorbed antitoxin from the original crude peptic antitoxin DP 1252 by a combination of absorption methods as indicated which shows a very close approach to the symmetrical curve of Fig. 7. An example of a purification to this degree is shown in Fig. 8, where the absorbed antitoxin was tested at the 20 u./ml. level against crude toxin. On comparing the curve for the crude antitoxin, Fig. 2, with the absorbed antitoxin derived from it,

* Since this paper was written the peptic fraction after absorption has been shown to give a very narrow symmetrical curve.

Fig. 8, it will be seen that for the same specific antitoxin concentration there has been marked change in the amount of floccules produced. It is not suggested that this absorbed antitoxin is now completely free from other antibodies, but there is good evidence that their concentration has been very considerably reduced as compared with the original peptic antitoxin.

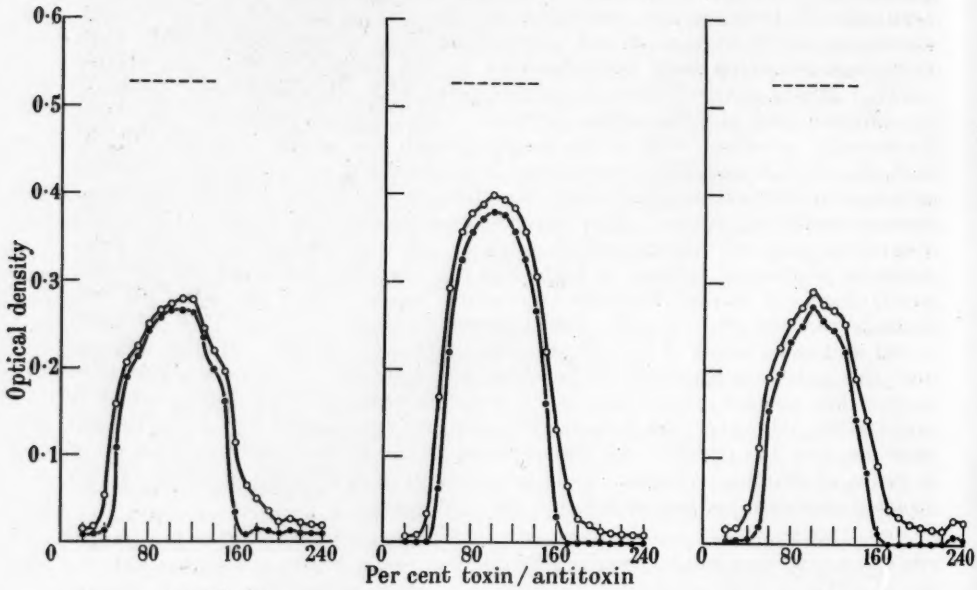

Fig. 6.—The reaction between absorbed antitoxin (T_4A) and purified toxin (2560 Lf/mg. P.N.). Dotted line = maximal density from Fig. 2. Other data as in Fig. 2.

Fig. 7.—Antitoxin (absorbed) from horse immunized with flash-heated toxoid. Fraction isolated between 30–50 per cent ammonium sulphate. Not pepsin treated. Dotted line = maximal density from Fig. 2. Other data as in Fig. 2.

Fig. 8.—The reaction between the peptic-antitoxin (after absorption with excess-iron antigens, peptic-toxin and *C. diphtheriae* suspension) and crude toxin. Dotted line = maximal density from Fig. 2. Other data as in Fig. 2.

Observations on the floccule precipitate.

It is not proposed to enter here into a detailed examination of the nature of the floccule precipitate produced by the interaction of diphtheria toxin and antitoxin, but two observations having a bearing on the work described are briefly mentioned.

We have found that if two drops, one of antitoxin and the other of toxin, both at a concentration of *ca.* 2000 u./ml., are placed on a slide and examined with a microscope, dust particles in each drop show Brownian motion. On mixing the two drops a gel is formed and all Brownian motion ceases at once.

This rapid production of a gel is very obvious at high concentrations but not so obvious at lower concentrations, for example, at 20 u./ml.

The second observation is concerned with the inclusion of particulate matter in the floccules themselves. To either antitoxin or toxin solutions of equal unit concentration (*e.g.*, 40 u./ml.) a little India ink (free from rapidly sedimenting particles) was added and then equal volumes of these were mixed ; the floccules produced entrained the ink and were black. Repeated washing with saline failed to remove the India ink from the floccules. When the experiment was repeated by mixing equal volumes of toxin and antitoxin first and then adding the India ink as rapidly as possible the floccules did not entrain the ink and washing with saline yielded almost colourless floccules. It is therefore clear that the reaction between antigen and antibody proceeds so rapidly that addition of the India ink as little as one second after mixing is too late to allow it to be incorporated in the gel structure. We were unable to show that the small amount of carbon forming the pigment in the ink absorbed any measureable amount of either antigen or antibody, and we were not therefore dealing with the precipitation of an adsorption-complex. This result is similar to that of Glenny and Okell (1924), who showed that a cholesterol suspension was precipitated with the toxin-antitoxin floccules and could act as an indicator in the toxin-antitoxin reaction.

The bearing of these observations on the composition of the toxin-antitoxin precipitate will be considered in the discussion.

DISCUSSION.

The observations of Pope *et al.* (1951) are incompatible with the findings of Pappenheimer and Robinson (1937). The former have shown that antitoxic sera contain many antibodies produced as the result of the injection into the horse of the crude culture filtrate from *C. diphtheriae*, and that in addition to the specific toxin and its antitoxin the floccules resulting from the interaction of " toxin " and " antitoxin " contain other antigen-antibody systems. The terms " toxin " and " antitoxin " are used here to denote the crude culture filtrate and the antitoxic serum derived from the horse. In many experiments the product we obtained by application of the method of Pope and Healey (1939) to washed floccules has always shown the presence of a series of antibodies with no antitoxic properties in addition to the specific antitoxin. The isolation of these antibodies from the well-washed floccules shows conclusively that the floccules do not consist only of the specific toxin-antitoxin complex. Since there is no reason to doubt that these other antigen-antibody systems are nitrogen-containing complexes, their presence in the floccules invalidates any conclusions regarding the specific toxin-antitoxin complex itself drawn from nitrogen determinations. However, Pappenheimer and Robinson regarded the floccules as specific floccules in the sense that their nitrogen was due solely to the specific diphtheria toxin and its antitoxin in the precipitate ; any other source of nitrogen would invalidate their conclusions. From their results they made deductions regarding the amount of nitrogen associated with one unit of antitoxin, and the nitrogen per Lf unit of toxin. Much use has been made of these values by other workers, and notably by Pappenheimer, Lundgren and Williams (1940) and Petermann and Pappenheimer (1941) in considering the reaction between toxin

and antitoxin. If these values are incorrect then the conclusions drawn would not be significant.

In the present work we have tried to purify antitoxic serum by absorbing antibodies other than antitoxin. Various materials have been studied for their antigen content and their ability to remove these non-antitoxic antibodies by forming antigen-antibody precipitates, but no ideal single source of all these antigens has been found. We have therefore had to rely on repeated absorption with different antigen systems, using the diffusion and optical methods to examine the product, in order to decide the degree of purification achieved. To obviate the criticism that any final product obtained represented only a small part of the original antitoxin, we have avoided methods which resulted in the loss of any appreciable amounts of the specific antitoxin. Thus, for example, in the absorption with peptic-toxin, at the T_4A stage the combined specific and mechanical losses did not exceed 20 per cent.

For the consideration of the results obtained by the optical method it is convenient to use the term " flocculation envelope " to describe the interaction between the antigens and antibodies. A comparison of the curve for the original antitoxin with those obtained after absorption shows that, for constant antitoxin concentration, there has been marked reduction in the amount of precipitate produced, and that much of the original precipitate was due to antibodies other than antitoxin. Clearly the shape of this envelope will depend on the reacting systems present, will change as absorption proceeds, and will not be constant until the final reaction is that of a single antigen-antibody system. Provided one antibody only, the specific antitoxin, is available in the serum, then it would be expected that the shape and area of the flocculation envelope would be constant irrespective of the number of antigens present in the crude toxin-filtrate or any fraction prepared from it. The absorbed antitoxins described here do not conform to this definition, and for this reason we do not consider that they are to be regarded as mono-antibody systems. This is largely due to the difficulty, which we have pointed out, of obtaining all the necessary antigens free from specific toxin. Thus, the excess-iron filtrates are obviously deficient in some antigens apart from the specific toxin, and it would appear that in the presence of excess iron in the culture filtrate the metabolism of *C. diphtheriae* is altered considerably ; toxin production is inhibited, but at the same time other changes appear in the composition of the culture filtrate. The washed *C. diphtheriae* suspensions are poor absorbents of the non-antitoxic antibodies ; the amounts required are too great to provide a practical method. *C. diphtheriae* grown for 3 days was better than that grown for 7 to 10 days for use as suspensions ; grinding the bacteria with sharp sand in a ball-mill appeared to increase their absorptive properties. Fortunately the peptic-toxin provides a reasonable source of antigens, and its deficiencies resulting from the destructive action of pepsin on some of the non-toxic antigens can be made good to a very large extent by combining the absorptive action of all three types of antigen.

When the original peptic antitoxin has been absorbed as completely as possible in this way, the amount of floccules produced at equal antitoxin concentrations in the respective 100 per cent mixtures is very obviously different even to the naked eye. After this exhaustive absorption, precipitation of the antitoxin with toxin yielded floccules which must have approached more closely a true toxin-antitoxin complex composition because application of the peptic method

(Pope and Healey, 1939) yielded antitoxin (90 per cent yield) with a purity of 211,000 u./g. protein. This antitoxin had been isolated from crude peptic antitoxin with an initial value of 16,000 u./g. protein. Pope and Healey (1939) reported the isolation of antitoxin from floccules with a value of 135,000 u./g. protein, and stated that all the protein appeared to be precipitable by toxin, but made no claim that it was pure antitoxin. At that time they were unable to show the presence of antibodies other than antitoxin ; the methods employed in this work were not available then. Unfortunately this material, prepared in 1938, was lost during the war, but all products of a similar purity isolated recently were contaminated with antibodies other than antitoxin. Petermann and Pappenheimer (1941) obtained antitoxin from floccules by the peptic method with a purity of the same order as that of Pope and Healey. Northrop (1942) reported the isolation of crystalline diphtheria antitoxin. He digested the toxin-antitoxin floccules with crystalline trypsin, and isolated in small yield a material which showed constant solubility and had a purity of 130,000–140,000 u./g. protein. The isolation of antitoxin with a very much greater purity, reported here, suggests that all these earlier preparations were contaminated with non-antitoxic antibodies and since these form precipitates with their corresponding antigens in the toxin, it is not surprising that all the protein in the isolated material appeared to be specifically precipitable. We have clear evidence, to be reported in detail later, that even the product with a purity of 211,000 u./g. protein is far from being immunologically pure antitoxin.

The shape of the curves obtained by the optical method showed that while they were irregular with crude and partially absorbed antitoxin, they tended to become more symmetrical in shape as the non-antitoxic antibodies were removed. In addition to a reduction in the amount of floccules produced the limits of flocculation became narrower. If we assume that in the flocculation envelope a series of antigens and their antibodies reacts, each in the form of a narrow symmetrical curve, we must account for the presence of both components, *i.e.*, antigen and its antibody, in the precipitate across a wide zone. We have shown that when mixed at high concentrations the antigen-antibody systems form a gel, and that particulate matter such as india ink present initially in one of the components is incorporated into the gel. Since the mechanism of precipitate formation appears to depend on the production of large polymers consisting of antigen and antibody, it is probable that at ratios of antigen-antibody considerably removed from that for optimal precipitation, these large molecules will be co-precipitated in the gel produced by another system at its optimum ratio. To illustrate our meaning we can consider a mixture containing 50 per cent of the equivalent quantity of toxin. With the crude peptic antitoxin a heavy precipitate appeared in this mixture. Almost all the initial antitoxin could be recovered from the precipitate and none from the supernatant by pepsin treatment. Using a thoroughly absorbed antitoxin, a similar mixture gave no precipitate ; since there were no floccules the supernatant now yielded the antitoxin on peptic treatment. Obviously the production of a precipitate at the 50 per cent point was dependent on the presence of non-antitoxic antibodies, which acting as the occluding gel carried down the partly polymerised but non-flocculating toxin-antitoxin complex. The broad equivalence zone observed by Pappenheimer and Robinson (1937) and Cohn and Pappenheimer (1949) for horse antitoxin was not found by these latter workers for the antitoxin in human, rabbit, guinea-pig and other sera.

It is possible that the main difference lies in the extent of the immunization used to produce these antitoxins. Cohn and Pappenheimer obtained symmetrical precipitation curves from these sera after absorption with excess-iron antigens. Our own results indicate that fully absorbed horse antitoxin will give a symmetrical flocculation curve, and that any departure from this indicates the presence of other antibodies.

The separation of a large amount of antibodies with no specific antitoxic properties from these low value peptic antitoxins makes it evident that their low u./g. purity is partly the result of production by the horse of a series of antibody-proteins whose behaviour in the peptic concentration process is similar to that of the antitoxin. We cannot yet say with certainty that all the protein in these low purity antitoxins can be accounted for as the sum of the specific antitoxin and the other antibodies, but the evidence is in favour of this.

The bearing of our results on the accuracy of the Ramon flocculation test for determining either toxin or antitoxin is a matter of some interest. Since this method was introduced by Ramon (1922) numerous workers have used it (see Glenny, 1931), and although in many cases there is good agreement between *in vivo* and *in vitro* values, there have been reports of values in marked disagreement. An outstanding example of a serum that showed an *in vivo*/*in vitro* ratio of about 5 was reported by Barr and Glenny (1938). By fractionation with ammonium sulphate they obtained a product with a ratio of nearly 20. No flocculation at a point corresponding to the animal value could be obtained. These workers have made available to us antitoxic serum from this horse; re-tested after 16 years the ratio has remained almost unchanged. On the basis of results given in this paper it was considered that this might be a case where the true toxin-antitoxin neutral point was well outside the "flocculation envelope." If this was so the amount of floccules produced at the *in vivo* point could be very small, and might escape detection in the usual test at a level of about 20 Lf u./ml. By carrying out a flocculation test at a level of 400 Lf u./ml. we have obtained a typical flocculation with a value in close agreement with the *in vivo* result. However, the amount of floccule precipitate given by 1500 units of antitoxin (*in vivo* value) with its equivalent of toxin was very meagre. Both the heavy floccule precipitate at the *in vitro* value and the meagre one at the *in vivo* value yielded only traces of antitoxin on pepsin treatment of the washed floccules. Almost all the toxin-antitoxin complex remained in the supernatant from which the antitoxin was recovered on peptic treatment. Our detailed study of this serum will be reported later; we mention these results here, because there are increasing grounds for believing that pure antitoxin will be found to be associated with much less protein than that indicated by earlier work.

We feel that some comment should be made on the fact that diphtheria toxin and antitoxin preparations had been obtained which appeared to be homogeneous as judged by electrophoresis, ultracentrifuge sedimentation, and diffusion methods (Pappenheimer, Lundgren and Williams, 1940; Petermann and Pappenheimer, 1941; Northrop, 1942; Rothen, 1942), although material of similar purity is quite heterogeneous when examined by immunochemical methods. Even the preparations isolated by Northrop (1942) which were obtained in crystalline form and which showed constant solubility would now appear, on the basis of evidence presented here, to be heterogeneous products, since we have isolated antitoxin with a higher specific activity per g. protein. It is generally recognized

that crystallinity of a protein is no guarantee of its purity, and we would also suggest that constant solubility measurements in terms of nitrogen in the two phases may be misleading. Where it is possible, as it is for diphtheria antitoxin, measurement of the specific activity might have given indications of the heterogeneity of the product. The wide range over which Northrop's purified antitoxin flocculated with purified toxin is quite at variance with the narrow-range meagre flocculation given by our antitoxin purified by absorption methods. Regarding the physical methods for determination of the homogeneity of a protein we would compare these with the properties of a number of wax record-blanks which would have the same diameter, weight, thickness and composition (physical constants), but these after being used for a " recording " would now have a specific property, *e.g.*, speech or music, not detectable as a change in physical constants but only by specific methods. Until both toxin and antitoxin have been isolated in a state where they will pass the test of immunological homogeneity we feel that there is little point in attempting to characterize them more completely or to describe the reaction between toxin and antitoxin.

SUMMARY.

The purification of diphtheria antitoxin by the absorption of non-antitoxic antibodies has been investigated. Evidence is presented to show that these other antibodies are present in peptic-concentrated antitoxin in considerable quantity and that their presence reduces the degree to which the specific antitoxin may be concentrated and purified. A large proportion of the so-called specific toxin-antitoxin precipitate is made up of antigen-antibody precipitates other than toxin-antitoxin complex. With progressive purification of the antitoxin by absorption methods, the amount of precipitate for a constant amount of antitoxin is markedly reduced and the shape of the flocculation curve becomes almost symmetrical. Antitoxin with a value of 211,000 u./g. protein has been isolated in this way, but it is not immunologically homogeneous.

Our thanks are due to Dr. F. V. Linggood and the staff of the Toxin Production Department for the preparation of large volumes of ultrafiltered toxins and excess-iron culture filtrates, to Dr. C. L. Oakley for the *in vivo* tests on the absorbed antitoxins and the immunization of a horse with flash-heated toxoid; also to Mr. N. H. Jenkins for nitrogen determinations. We also express our thanks to Mr. A. T. Glenny, F.R.S. for very helpful discussions during the course of this work.

REFERENCES.

BARR, M., AND GLENNY, A. T.—(1938) *J. Path. Bact.*, **47**, 27.
BOWEN, H.—(1952) *J. Immunol.*, **68**, 429.
COHN, M., AND PAPPENHEIMER, A. M.—(1949) *Ibid.*, **63**, 291.
DOTY, P., AND EDSALL, J. T.—(1951) 'Advances in Protein Chemistry,' vol. vi. New York, N.Y.: (Academic Press Inc.), p. 95.
GITLIN, D., AND EDELHOCH, H.—(1951) *J. Immunol.*, **66**, 67.
GLENNY, A. T.—(1931) ' System of Bacteriology in Relation to Medicine.' London (Medical Research Council), vol. 6.
Idem AND OKELL, C. C.—(1924) *J. Path. Bact.*, **27**, 187.
GOLDBERG, R. J., AND CAMPBELL, D. H.—(1951) *J. Immunol.*, **66**, 79.

HARMS, J.—(1948) *Biochem. J.*, **42**, 390.
HARTRIDGE, H.—(1915) *J. Physiol.*, **49**, 406.
LINGGOOD, F. V., AND FENTON, E. L.—(1947) *Brit. J. exp. Path.*, **28**, 354.
MARKHAM, R.—(1942) *Biochem. J.*, **36**, 790.
MORGAN, W. T. J.—(1937) *Ibid.*, **31**, 2003.
NORTHROP, J. H.—(1942) *J. gen. Physiol.*, **25**, 465.
OAKLEY, C. L., AND FULTHORPE, A. J.—(1953) *J. Path. Bact.*, **65**, 49.
PAPPENHEIMER, A. M., AND JOHNSON, S. J.—(1937) *Brit. J. exp. Path.*, **18**, 239.
Idem, LUNDGREN, H. P., AND WILLIAMS, J. W.—(1940) *J. exp. Med.*, **71**, 247.
Idem AND ROBINSON, E. S.—(1937) *J. Immunol.*, **32**, 291.
PETERMANN, M., AND PAPPENHEIMER, A. M.—(1941) *J. phys. Chem.*, **45**, 1.
POPE, C. G.—(1932) *Brit. J. exp. Path.*, **13**, 207.—(1939*a*) *Ibid.*, **20**, 132.—(1939*b*) *Ibid.*, **20**, 201.
Idem AND HEALEY, M.—(1938) *Ibid.*, **19**, 397.—(1939) *Ibid.*, **20**, 213.
Idem, STEVENS, M. F., CASPARY, E. A., AND FENTON, E. L.—(1951) *Ibid.*, **32**, 246.
RAMON, G.—(1922) *C.R. Soc. Biol., Paris*, **86**, 661.
ROTHEN, A.—(1942) *J. gen. Physiol.*, **25**, 487.
TINSLEY, H.—(1941) Brit. Patent, No. 537784.
TREVAN, J. W.—(1926) *Biochem. J.*, **19**, 1111.

THE UPTAKE OF RADIOACTIVE PHOSPHORUS BY THE SKIN OF THE RABBIT.

H. J. WHITELEY, H. B. STONER* AND C. J. THRELFALL.

From the Department of Pathology, University of Sheffield.

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IT has been shown by Whiteley and Ghadially (1951) that hair growth does not occur uniformly over the rabbit trunk, but in bilateral zones of intense growth while the remainder of the skin is quiescent. This hair growth is associated with considerable enlargement of the hair follicles, which extend deeply into the dermis, and show a much increased mitotic activity in their deeper parts (Whiteley and Ghadially, 1952). There was, however, no significant difference in mitotic activity in the epidermis over the two zones. In view of the great difference in follicular activity in the two zones it was thought that there might also be a difference in their uptake of ^{32}P and preliminary studies showed that this was so. More ^{32}P was taken up by the growing zone than by the quiescent zone. As many factors such as increased blood supply, increased cellular density and activity in the growing zone could play a part in this it was decided to investigate the problem.

METHODS.

Thirty-six Agouti and Chinchilla rabbits of either sex and between 1 and 2 kg. body wt. were examined for zones of hair growth, most conveniently by blowing the hair apart over the trunk, where the dark zones of hair growth were easily observed. The hair was then removed with clippers from a band encircling the trunk, and the animal left for 24–48 hr. to allow any inflammatory reaction, due to the clipping, to subside. Carrier-free ^{32}P, obtained from the Atomic Energy Research Establishment, Harwell, was diluted with phosphate-saline buffer, pH 7·0 (Harrison and Raymond, 1951) to give a solution containing 1·0 mc. ^{32}P per 10 ml. which was assayed as described by Stoner, Threlfall and Green (1952). The dose of ^{32}P was 75 μc. per kg. body wt. given intravenously. The rabbits were killed at intervals after the injection of ^{32}P by the intravenous injection of nembutal. The skin of the trunk, including the panniculus carnosus, was removed immediately, pinned out under even tension with the internal surface uppermost and dried overnight at 37°. Equal areas from the growing and quiescent zones were punched out of the dried skin with a cork borer (5 mm. diam.) and dissolved in 1·0 ml. 5N NaOH. These extracts were diluted to 10·0 ml. and their radioactivity determined in a M6 liquid counter (Veall, 1948). Contact autoradiographs of the dried skin were prepared on " Kodirex " film, the internal surface of the skin being in contact with the emulsion. The exposure varied from 24 to 48 hr., depending on the initial radioactivity as determined with an Airmec radiation monitor. The films were developed to maximum density in Kodak D 19b developer under standard conditions. The transmission of the developed films was determined photoelectrically with a Hilger " Biochem " absorptiometer, placing a circular iris (7·0 mm. diam.) directly in front of the cell, between it and the film. The density of the different areas was calculated from the formula

$$\text{Density} = \log_{10} \frac{I_0 \text{ (unexposed transmission)}}{I_E \text{ (exposed transmission)}}.$$

* Member of the External Scientific Staff of the Medical Research Council.

One rabbit, with good growth zones, was given 1 μc. ^{32}P per g. body wt. intravenously and killed 24 hr. later. Skin from growing and quiescent zones was fixed in ethanol. Paraffin sections (7 μ thick) were cut from these samples and autoradiographs prepared by the stripping film technique. This was very kindly done for us by Dr. L. F. Lammerton of the Chester Beatty Institute.

To study the distribution of ^{32}P in the phosphate fractions of the skin, extracts were prepared from undried skin removed as described above. Equal areas were cut out with a cork borer (15 mm. diam.) as quickly as possible and frozen in liquid air. The samples were then weighed and extracted with 6 volumes ice-cold 10 per cent trichloroacetic acid by grinding with quartz till an even paste was produced. This was centrifuged at 1800 g for 10 min. at 0°. The total acid-soluble P was determined by the method of Fiske and SubbaRow (1925). The acid-insoluble P was fractionated by Schmidt and Thannhauser's method (1945), P again being determined by the method of Fiske and SubbaRow. The radioactivity of the fractions was determined with a M6 liquid counter and expressed as counts per min. per mg. P (specific activity).

The blood supply to the growing and quiescent zones was investigated in sections stained by Pickworth's method (Mallory, 1938) and by the use of ^{32}P-tagged red cells. Cross-matched rabbit red cells (packed volume 8–10 ml.) were tagged with ^{32}P as described by Reeve and Veall (1949), diluted to the original volume with 0·9 per cent NaCl and injected intravenously. The rabbits were killed 10 min. after injection and autoradiographs (14 days' exposure) prepared from the dried skin as before. To obtain quantitative data on the amount of blood in the different zones of the skin, areas were removed before drying with a cork borer (15 mm. diam.), taking care to avoid obvious blood vessels. These samples were dissolved, with heat, in 5·0 ml. conc. HNO_3, and the radioactivity of the extract determined after dilution to 10·0 ml., with a M6 liquid counter, a correction being made for the specific gravity of the solution. The blood content was then calculated from the counts per min. per ml. blood determined on 1·0 ml. samples taken when the rabbit was killed and suitably diluted with 0·02 N HCl. The plasma, at the time of death, contained less than 1·0 per cent of the radioactivity of the whole blood. The blood content of the skin was also determined by the method of Lowry and Hastings (1942).

The extra-cellular space in the two zones of the skin was studied with $^{35}SO_4''$. The extra-cellular distribution of injected SO_4'', in acute experiments, has been demonstrated by Greenwald (1918), Bourdillon and Lavietes (1936) and Smith, Winkler and Schwartz (1939). Radioactive sulphate has recently been used to determine the extra-cellular space by Walser (1952), and the rapid equilibration of the ion about the capillary membrane has been shown by Scheatz and Wilde (1950). Carrier-free $^{35}SO_4''$ was obtained from the Atomic

DESCRIPTION OF PLATES.

FIG. 1.—Composite photograph of the dried skin (L) and the autoradiograph as a mirror image, showing the close correlation between the actively growing skin and the areas of increased photographic density from a rabbit killed 5 min. after 75 μc. ^{32}P per kilo body wt. given intravenously. Density ratio, 1.6; radioactivity ratio, 1.76. Note that the blood vessels visible on the dried skin do not register on the autoradiograph.

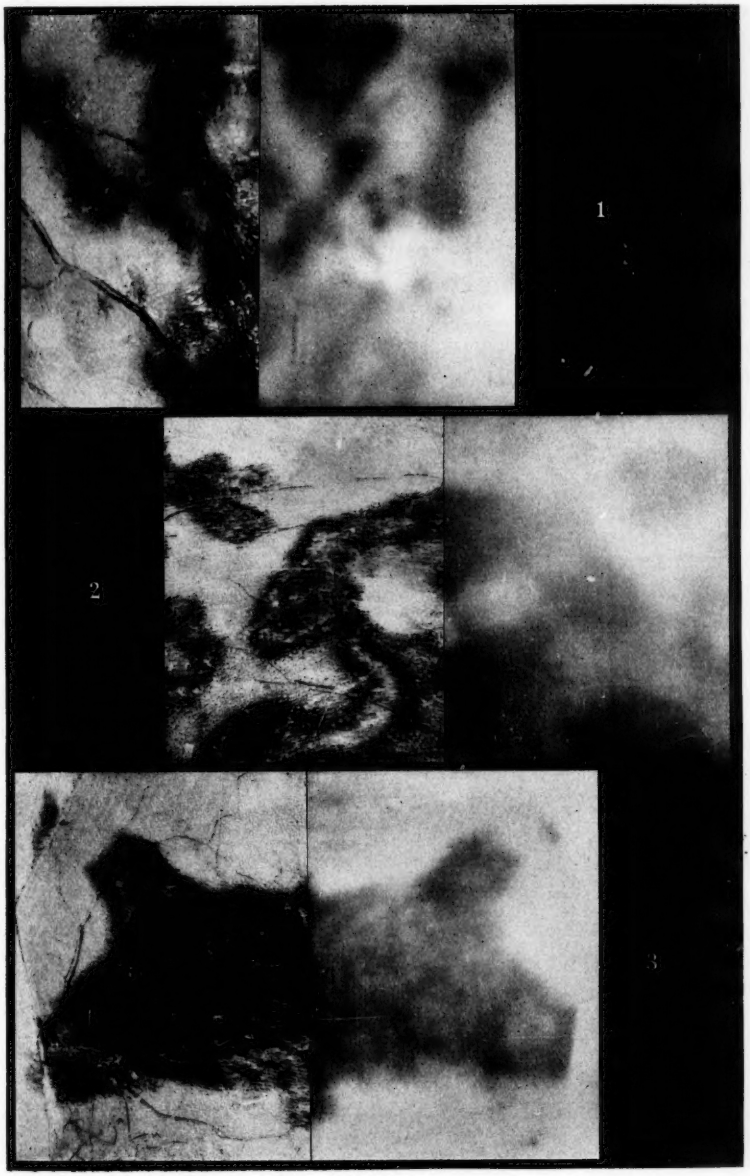
FIG. 2.—Composite photograph of the dried skin and the autoradiograph from a rabbit killed 1 hr. after 75 μc.^{32}P per kg. body wt. given intravenously. Density ratio, 2.8; radioactivity ratio, 2.7.

FIG. 3.—Composite photograph of the dried skin and autoradiograph from a rabbit killed 24 hr. after 75 μc.^{32}P per kg. body wt. given intravenously. Density ratio, 2.9; radioactivity ratio, 2.9.

FIG. 4.—Composite photograph of the skin and autoradiograph from a rabbit killed 10 min. after the intravenous injection of cross-matched ^{32}P tagged red cells. The larger vessels stand out clearly and there is a slightly increased density corresponding to the zone of hair growth.

FIG. 5.—× 700. Stripping film autoradiograph from deeper part of hair follicle in growing zone. Note the large number of developed granules over the follicles. Rabbit killed 24 hr. after 1 μc.^{32}P per g. body wt. given intravenously.

FIG. 6.—× 700. Stripping film autoradiograph of a follicle in quiescent zone from the same rabbit as in Fig. 6. Few developed granules visible.



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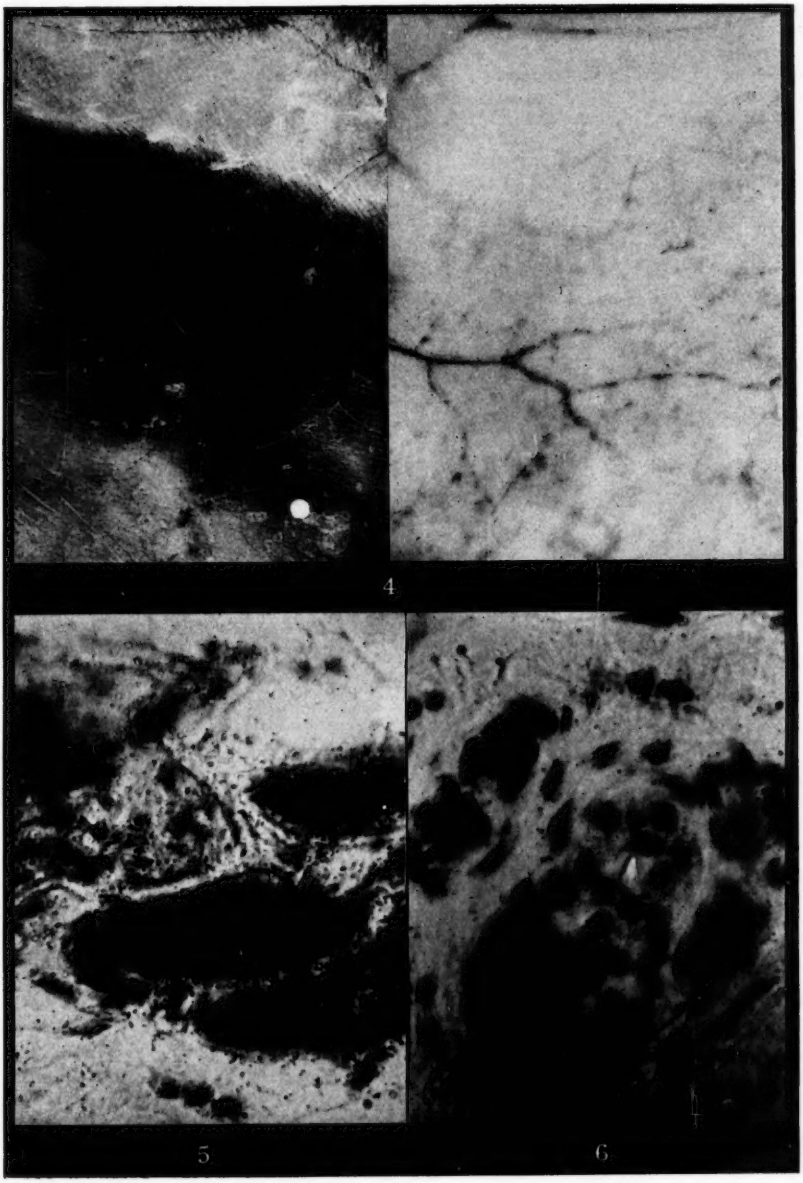

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Energy Research Establishment, Harwell, and diluted with 0·9 per cent NaCl to give a solution containing 100 μc. per ml. Rabbits were given 75 μc. $^{35}SO_4''$ per kg. body wt. intravenously and 30 min. allowed for equlilibration. They were then killed and the skin removed as above. Areas of skin were cut out with a cork borer; the skin samples were weighed and dried for 48 hr. at 110° before extraction (Eggleton, 1952). The dried skin was extracted with successive amounts of 10 per cent trichloroacetic acid (total—20 ml. per g. wet wt.), carrier sulphate being added at the beginning of the extraction. The extracts were combined and the sulphate precipitated as benzidine sulphate. This was collected and dried and aliquots were plated out on stainless steel planchettes. These were counted with an end-window counter (G.E.C. Type EHM 2, window 1·9 mg. per cm.^{2}) a correction being made for self-absorption. Just before the rabbit was killed a sample of blood was taken by cardiac puncture and an aliquot of the plasma, after removal of the proteins with 10 per cent trichloroacetic acid, was similarly treated to determine the counts per min. per ml. plasma. From these data the volume of distribution of $^{35}SO_4''$ in the skin could be calculated.

RESULTS.

Autoradiographs of whole skin.

In 3 rabbits killed 5 min. after the intravenous injection of ^{32}P it was found that much larger amounts of the isotope had been taken up by the growing areas of the skin than by the quiescent areas (Fig. 1). The zone of hair growth was accurately depicted as a dense shadow on the autoradiograph. The ratio of the densities of the two zones was between 1·5 and 1·65 and that of the total radioactivity of equal areas of skin was 1·8.

In 9 rabbits killed 1 hr. after the injection of ^{32}P a similar picture was seen, more isotope having been taken up by the actively growing skin (Fig. 2). The average ratio of the densities of the two zones was 2·4 (range 2·0–2·8) in the 7 animals in which it was measured. This figure agrees well with the ratio of the total flux from the two areas (Table IV), which was 2·3 at this time.

The difference in the behaviour of the growing and quiescent zones was still apparent 24 hr. (Fig. 3) and 72 hr. after the injection of ^{32}P. Throughout the experimental period there was an increasing differentiation between the two zones on the autoradiograph as shown by the increasing ratio of the densities, which was 2·7 (mean of 4 animals) at 24 hr. and 3·4 at 72 hr. The agreement between the ratio of the densities of the two zones and the ratio of their radioactivity, as measured by other methods, was maintained, for the ratio of the total fluxes at 24 hr. was 2·9 (Table IV).

Blood content and extra-cellular space of the skin.

The intravenous injection of " tagged " red cells, 10 min. prior to the animal being killed and subsequent autoradiography of the dried skin, revealed that there was an increased blood supply to the area of active growth (Fig. 4). This was confirmed by determining the blood content of the skin after giving tagged red cells (Table I). The growing zone contained more blood whether the comparison was made on the basis of equal area or equal weight. It was, therefore, an absolute increase, unrelated to the greater thickness of the skin in the growing zone. Our results with this method were more constant than those obtained by Watts (1951) in rats, perhaps due to his failure to take the hair growth zones into account. The method was checked by the spectrophotometric determination of the blood in growing and quiescent zones of skin, as described by Lowry and Hastings (1942), which gave results of the same order. Pickworth staining of

TABLE I.—*Blood Content of Growing and Quiescent Zones of Rabbit's Skin in Relation to Area and Weight.*

Blood content of skin.			
ml. per sq. cm.		ml. per g. wet wt.	
Quiescent zone.	Growing zone.	Quiescent zone.	Growing zone.
0·0003 ; 0·0003	0·0007 ; 0·0008	0·0038 ; 0·0039	0·0064 ; 0·0073
0·0003 ; 0·0007	0·0016 ; 0·0016	0·0026 ; 0·0048	0·0082 ; 0·0087
0·0003 ; 0·0004	0·0010 ; 0·0010	0·0040 ; 0·0043	0·0066 ; 0·0064
0·0004 ; 0·0003	0·0009 ; 0·0013	0·0091 ; 0·0064	0·0088 ; 0·0126
0·0005 ; 0·0004	0·0010 ; 0·0010	0·0041 ; 0·0045	0·0061 ; 0·0060
Mean 0·0004	0·0011	0·0048	0·0077
S.D. 0·0001	0·0003	0·0018	0·0020

The duplicate determinations were made on separate samples.

thick sections (200 μ) from the two zones showed that the increased blood supply in the growing skin was due to an increase in the number of capillaries around the bases of the growing hair follicles. Despite these findings it should be noted that after the intravenous injection of ^{32}P solutions the major blood vessels were only occasionally seen as positive shadows on the early autoradiographs. They sometimes appeared as negative shadows on the later ones. This may be related to the density of the vessel wall.

The extra-cellular space (interstitial space + plasma space) of the skin was determined with $^{35}SO_4''$ in 5 animals. The absolute figures obtained were lower than those given for rabbit skin by Harrison, Darrow and Yannet (1936) and Manery and Hastings (1939), probably because the recovery of the added carrier sulphate was not complete (70–92 per cent). If allowance was made for this the values were of the same order as theirs. However, as the degree of recovery from the two zones was the same in any particular experiment, it was possible to utilize the ratio of the values for the two zones as a measure of the difference in their extra-cellular spaces. The ratio of the extra-cellular space of the growing zone to that of the quiescent zone was 1·76 (range 1·5–2·0) for equal areas of skin, the same as that between the wet weights of equal areas of skin from the two zones (*vide infra*). The extra-cellular space ratio between equal weights of growing and quiescent skin was 0·99 (range 0·90–1·06), making it clear that the greater extra-cellular space of the growing zone is merely a reflection of its greater thickness. Consequently, the absolute increase in the blood content of the growing zone must have occurred at the expense of the interstitial space.

Phosphate distribution in rabbit skin.

The amount of P in the acid-soluble and nucleic acid fractions of the growing and quiescent zones is shown in Table II. There is no difference in the total acid-soluble P concentration in the two zones when the results are expressed per unit weight of skin, but there is a very significant difference when they are expressed in terms of a unit area of skin. This is a reflection of the greater thickness and cellularity of the growing zone, which is also illustrated by its greater weight (wet) per cm.^{2}, which is 147 mg. (S.D. 27), as opposed to 84 mg. (S.D. 22) per cm.^{2} in the quiescent zone (calculated from duplicate determinations in 19 rabbits). Although the total acid-soluble P concentration is not affected

TABLE II.—*Distribution of Phosphorus in Rabbit Skin.*

	Total acid-soluble P.				Total nucleic acid P.			
	Growing zone.		Quiescent zone.		Growing zone.		Quiescent zone.	
	mg. P per g. wet wt.	mg. P per cm².	mg. P per g. wet wt.	mg. P per cm².	mg. P per g. wet wt.	mg. P per cm².	mg. P per g. wet wt.	mg. P per cm².
	0·50	0·07	0·47	0·04	0·42	0·06	0·28	0·02
	0·59	0·10	0·45	0·04	0·45	0·08	0·24	0·02
	0·44	0·06	0·42	0·05	0·33	0·05	0·19	0·02
	0·46	—	0·38	—	0·40	—	0·27	—
	0·45	0·06	0·55	0·03	0·48	0·07	0·30	0·02
	0·49	0·07	0·50	0·04	0·52	0·08	0·34	0·02
	0·55	0·05	0·46	0·03	0·50	0·05	0·24	0·01
	0·49	0·08	0·55	0·05	0·53	0·09	0·24	0·02
	0·49	0·07	0·55	0·05	0·53	0·08	0·30	0·03
Mean	0·50	0·07*	0·48	0·04	0·46*	0·07*	0·27	0·02

* = Significant difference between corresponding means at level P = 0·01.

by the activity of the cells the nucleic acid P concentration is, for the level in the growing zone is significantly higher than in the quiescent zone whether the results are expressed in terms of unit area or unit weight. This is chiefly due to an increase in the ribose nucleic acid fraction.

The specific activity of both phosphate fractions was greater in the growing zone 1 hr. after ^{32}P injection, but after 24 hr. this difference in the total acid-soluble P had disappeared while becoming more marked in the nucleic acid P (Table III).

TABLE III.—*Specific Activity of the Phosphorus Fractions in Rabbit's Skin Sampled 1 hr. and 24 hr. after the Intravenous Injection of 75 μc. ^{32}P per kg. Body Wt.*

	Specific activity (counts per min. per mg. P).			
	Total acid soluble P.		Total nucleic acid P.	
	Growing zone.	Quiescent zone.	Growing zone.	Quiescent zone.
1 hr. after ^{32}P injection	29,300	20,500	2,070	1,810
	37,600	34,800	1,650	1,210
	18,600	13,900	750	750
	21,400	15,100	770	575
	27,200	21,400	1,440	1,250
Mean	26,800	21,100	1,340	1,120
V.R.*	33·2†		13·3‡	
24 hr. after ^{32}P injection	10,500	8,550	8,400	4,080
	8,950	8,850	9,990	5,680
	7,420	6,880	4,900	2,950
	8,200	7,500	5,880	2,070
Mean	8,770	7,950	7,290	3,700
V.R.*	4·3		41·0†	

* = variance ratio $= \frac{(\Sigma d)^2}{n} \Big/ \frac{1}{n-1}\left[\Sigma d^2 - \frac{1}{n}(\Sigma d)^2\right]$,

where d = difference in the specific activities of the growing and quiescent skin in each rabbit;
n = number of animals;
† = significant at level P = 0·01;
‡ = significant at level P = 0·05;

From the data in Tables II and III the total electron flux from the two zones of the rabbit skin could be calculated according to the formula :

$$\text{Total electron flux} = \text{counts/min./mg. P} \times \text{mg. P/cm.}^2 \times \frac{100}{\text{Geiger tube efficiency}}$$

with the results shown in Table IV. It is interesting that after 24 hr. the flux from the nucleic acid P forms a much greater proportion (46 per cent) of the total

TABLE IV.—*Electron Flux from Rabbit Skin 1 hr. and 24 hr. after the Intravenous Injection of 75 μc. ^{32}P per kg. Body Wt.*

Time after ^{32}P injection.	Fraction.	Disintegrations per min. per cm.^{2}	
		Growing zone.	Quiescent zone.
1 hr.	Total acid-soluble P	27,000	12,200
	Total nucleic acid P	1,300	300
	Total P	28,300	12,500
24 hr.	Total acid-soluble P	8,700	4,600
	Total nucleic acid P	7,300	1,000
	Total P	16,000	5,600

Data calculated from the mean values for the total acid-soluble P and total nucleic acid P and their mean specific activities. Efficiency of the Geiger tube 7 per cent.

flux from the growing zone than at 1 hr., when it provides only 5 per cent of the total. This increase in the significance of the contribution from the nucleic acid P is also seen in the quiescent zone, but to a less extent. The ratio between the total electron flux from the growing and quiescent zones after 1 hr. is 2·3 and after 24 hr. 2·9, confirming the increased differentiation between the two zones after 24 hr. seen in the autoradiographs. The total flux fell during the 24 hr., due to the more rapid turnover of the acid-soluble P.

The eventual localisation of the ^{32}P in the follicles of the growing zone was confirmed by stripping film autoradiographs of sections of skin in the rabbit killed 24 hr. after the intravenous injection of 1 μc. per g. body wt. The concentration of developed granules was found to be very high over the deepest parts of the follicles in the growing zone (Fig. 5), while in the quiescent zone (Fig. 6) the concentration of developed granules was low and similar to that of the connective-tissue stroma in both zones.

DISCUSSION.

Our results show that the uptake of ^{32}P by rabbit skin is dependent on its physiological state. Areas of active hair growth take up more ^{32}P than quiescent zones, and this difference is visible on contact autoradiographs. Several factors contribute to this effect, and it is only after the cells have been exposed to ^{32}P for 24 hr. or more that the differentiation between the two zones can be attributed to a fundamental metabolic difference. Then, and later, the increasing differentiation between the two zones is due to the greater concentration and higher specific

activity of the nucleic acid P in the growing zone. The higher specific activity of this fraction is, biologically, the most outstanding difference between the two zones in their behaviour towards ^{32}P. This difference can be seen as early as 1 hr. after the injection, but its contribution to the total effect at that time is very small.

At shorter intervals after the ^{32}P injection the very evident differentiation between the two zones is mainly due to other factors. It has been shown that, bearing in mind the greater weight per unit area of the growing zone, there is an absolute increase in the blood content of this zone with a relative increase in the extra-cellular space and acid-soluble P content per unit area. The ratios (growing zone/quiescent zone) of the weight, extra-cellular space and acid-soluble P content per unit area of skin are all about 1·8, and this is also the ratio of their radioactivities 5 min. after the intravenous injection of ^{32}P. This suggests that the ^{32}P is distributed through the extra-cellular space by this time, since the ratio of the blood content per unit area is 2·8. It is likely that when the skin is examined so soon after the injection the difference on the autoradiograph is mainly due to the greater extra-cellular space per unit area of the growing zone.

One hour after the injection the main source of the flux from each zone is the acid-soluble P. If ^{32}P was equally distributed to the acid-soluble P fraction the ratio of the radioactivities of the two zones would still be the same as that of their weights per unit area, but the ratio at 1 hr. is considerably higher than this, due to the greater specific activity of the acid-soluble P in the growing zone. This may represent a further metabolic difference between the zones, but one cannot be certain of this until the components of the acid-soluble P fraction have been accurately separated. This has not yet been possible.

It was hoped that a method could be developed from this autoradiographic technique which would be suitable for the study of the effect of local and general conditions on the metabolism of the skin. The technique may prove useful if the various factors discussed above are kept in mind. It is important to realize that inhibition of nucleic acid synthesis cannot be postulated unless it can be shown that the passage of ^{32}P into the cells of the follicle is unaffected. At present we are studying the distribution of ^{32}P in the skin of the trunk after hind limb ischaemia when the difference between the two zones is not seen on the autoradiograph.

SUMMARY.

The distribution of ^{32}P in the skin of the rabbit after intravenous injection has been studied and it was found that skin showing active hair growth contained more of the isotope than did skin in which the hair follicles were quiescent. This was a constant finding whether the skin was examined 5 min. or 72 hr. after the injection, but the differentiation between the two zones increased with time.

Several factors contribute to the greater radioactivity of the growing zone and the importance of any one of them depends on the time which has elapsed after the ^{32}P injection. Shortly after the injection the greater extra-cellular space per unit area in the zone of active hair growth is important, whereas, an hour after the injection, the difference is mainly due to the larger amount of acid-soluble P per unit area of the growing zone and its higher specific activity. Later (> 24 hr.) the greater concentration and higher specific activity of the nucleic acid P in the growing zone play an increasingly important part.

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REFERENCES.

BOURDILLON, J., AND LAVIETES, P. H.—(1936) *J. clin. Invest.*, **15**, 301.
EGGLETON, M. G.—(1952) *J. Physiol.* **117**, 172.
FISKE, C. H., AND SUBBAROW, Y.—(1925) *J. biol. Chem.*, **66**, 375.
GREENWALD, I.—(1918) *J. Pharmacol.*, **11**, 281.
HARRISON, H. E., DARROW, D. C., AND YANNET, H.—(1936) *J. biol. Chem.*, **113**, 515.
HARRISON, G. E., AND RAYMOND, W. H. A.—(1951) *Brit. med. J.*, ii, 930.
LOWRY, O. H., AND HASTINGS, A. B.—(1942) *J. biol. Chem.*, **143**, 257.
MALLORY, F. B.—(1938) 'Pathological Technique.' Philadelphia (W. B. Saunders), p. 257.
MANERY, J. F., AND HASTINGS, A. B.—(1939) *J. biol. Chem.*, **127**, 657.
REEVE, E. B., AND VEALL, N.—(1949) *J. Physiol.*, **108**, 12.
SCHEATZ, G. C., AND WILDE, W. S.—(1950) *Amer. J. Physiol.*, **162**, 687.
SCHMIDT, G., AND THANNHAUSER, S. J.—(1945) *J. biol. Chem.*, **161**, 83.
SMITH, P. K., WINKLER, A. W., AND SCHWARTZ, B. M.—(1939) *Ibid.*, **129**, 51.
STONER, H. B., THRELFALL, C. J., AND GREEN, H. N.—(1952) *Brit. J. exp. Path.*, **33**, 398.
VEALL, N.—(1948) *Brit. J. Radiol.*, **21**, 347.
WALSER, M.—(1952) *Proc. Soc. exp. Biol., N.Y.*, **79**, 372.
WATTS, R. W. E.—(1951) *J. Physiol.*, **114**, 382.
WHITELEY, H. J., AND GHADIALLY, F. N.—(1951) *Brit. J. Cancer*, **5**, 353.—(1952) *J. Path. Bact.*, **64**, 651.

AN IMMUNO-CHEMICAL STUDY OF ISOLATED SERUM PROTEIN FRACTIONS.

C. WUNDERLY, ELIZABETH GLOOR* AND A. HÄSSIG.

From the Zürich University Medical Clinic and the Department of Bacteriology and Serology, Central Laboratory of the Blood Transfusion Service of the Swiss Red Cross, Berne.

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GOETTSCH and Reeves (1936) showed by a quantitative immuno-chemical method that the serum proteins in nephrosis differ immunologically from those in normal individuals; salt fractionation was used for isolation of the proteins. Alving and Mirsky (1936) used ammonium sulphate for the separation of the protein fractions in nephrosis and found that the cystine content of the albumin was lowered; similarly Albanese, Davis, Smetak and Lein (1949) found an abnormal distribution of amino-acids in the plasma proteins. Recent comparison of salt fractionation with separation by electrophoresis has shown that the Howe technique precipitated not a pure albumin, but most of the α_1 and even some of the α_2 globulins as well, and that in sera with reduced albumin content the precipitation becomes less quantitative than in normal serum. Deutsch, Alberty, Gosting and Williams (1947) found that rabbit antiserum prepared by three intraperitoneal injections of 100 mg. human γ-globulin also reacted with human albumin, and concluded that even slight impurities present in the immunising antigen could give rise to corresponding antibodies. Jager, Smith, Nickerson and Brown (1948) demonstrated that antibody to the human γ-globulin fractions II—1, 2 and II—3 (alcohol precipitation) reacts not only with the γ-globulin which is estimated electrophoretically but with about 10 per cent of a protein belonging to the α- or β-globulin; thus immunological evaluation of γ-globulin gave higher results than the electrophoretic estimations. Better separation can be obtained with the ethanol-precipitation technique; highly purified human and bovine plasma protein fractions have been prepared which corresponded more closely with their immuno-chemical identification whether they were prepared from serum, urine, ascitic fluid or oedema fluid. Thus Gitlin and Janeway (1952) showed that the albumin of 11 nephrotic children was immuno-chemically indistinguishable from crystallised normal human serum albumin. Albumin concentrations measured by precipitation with horse antiserum were in better accord with electrophoretic findings than those obtained with rabbit antiserum.

The accuracy of immunological determinations has been increased by the use of spectrophotometric methods for determining total and antibody protein in specific precipitates. For the quantitative measurement of the protein absorption Eisen (1948), Gitlin (1949) and Wunderly and Hässig (1949) used the Beckman spectrophotometer. The sensitivity was compared with various other methods

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by Kunkel and Ward (1950); they found that 0·09 mg. protein is needed to give an optical density reading of 0·07 in the Beckman spectrophotometer on wavelength 287 mμ, whereas only 0·02 mg. protein was needed when the ninhydrin method of Moore and Stein (1948) was used. Both these procedures have the advantage of requiring much smaller quantities of antiserum, a factor which is often decisive when, in order to make conditions constant, one antiserum must be used for the entire series of tests. Thus Chow (1947) needed 2 ml. antiserum for each precipitation, but the new procedures require only 0·2 ml.

In the present investigation serum proteins were separated by preparative paper-electrophoresis (Hugentobler, Wunderly and Schneider, 1951). Fractions of normal human serum were compared by immuno-chemical characterisation with those in various pathological sera.

METHODS.

Antigens.—Normal human serum was supplied by the Central Laboratory for the Blood Transfusion Service of the Swiss Red Cross at Berne; pathological sera came from the Zürich University Clinic. All sera were centrifuged in a fresh state and then kept frozen at − 32°.

Paper electrophoresis.—The total concentration of protein in the sera was measured by the refractive index and often controlled by N estimation by micro-Kjeldahl. The colloid lability of the sera was estimated by the cadmium reaction (Wunderly and Wuhrmann, 1947).

The serum proteins were separated by paper electrophoresis (Hugentobler *et al.*, 1951). Thick filter-paper was used which yielded good separation, and the material obtained from two paper strips was usually sufficient for the ensuing immuno-chemical estimation. On each of two filter-paper strips of 54 × 7 cm. Munktell paper No. 2/350 (Sweden) serum was placed in a thin line so that 25–30 mg. protein was deposited per paper. When the α-globulin content was low 3 and even 4 paper strips had to be used to obtain the necessary amount of protein. All the electrophoreses were carried out in a closed chamber (Wunderly and Reynaud, 1952); this also reduced the risk of contamination. Twenty-four hr. electrophoresis at pH 8·7 (barbitone-Na barbitone buffer of I 0·06) 185 V. and 14–16 mA. separated the fractions sufficiently (see Fig. 1). Along one of the two papers a strip of 2 cm. width was cut off, dried and stained with bromphenol blue. The staining revealed the exact positions of the fractions, and the guide strip was then placed beside the unstained papers from which the four fractions were cut off accordingly. The wet papers were immediately placed in 8–10 ml. 0·85 per cent saline and remained there at 2° for 6–8 hr. They were then squeezed and the result was that about 75 per cent of the protein had been removed from the paper (Kunkel and Tiselius, 1951). These eluted fractions were dialysed against tap water and distilled water, and then concentrated by dialysis against a 15 per cent solution of high molecular dextran until each fraction was contained in a volume of 1–2 ml. Colloidal solutions of β- and γ-globulin have the tendency to become cloudy and can be solubilised again with crystals of NaCl. From each fraction 0·1 ml. was diluted with 3 ml. 0·1 N NaOH and the ultra-violet absorption was measured in the Beckman spectrophotometer at λ 280 mμ. By diluting with saline where necessary the globulin fractions were brought to 0·080 and the albumin to 0·050 optical density. According to their specific absorption the fractions then contained 190 mg. per cent protein and were ready for precipitation. In order to compensate for possible time effects (Beaven and Holiday, 1950) ultra-violet absorption was always measured directly after solubilisation in 0·1 N NaOH.

Antibodies.—Three rabbits were given 6 intravenous injections of 0·5 ml. normal human serum (group O) at intervals of 2, 2, 2, 2 and 3 days. From the 13th to 16th day following immunisation about 15 ml. blood was taken daily from the ear vein. The sera were pooled, divided into lots of 2 ml. and kept frozen at − 32°. Before use the antiserum was centrifuged for 5 min. at 7500 r.p.m.

Precipitation.—Pilot experiments with serial dilutions of the different protein fractions were carried out in order to establish the optimal zone of precipitation. In all pilot experiments the supernatant fluids were tested for excess of antibody and antigen. It was re-

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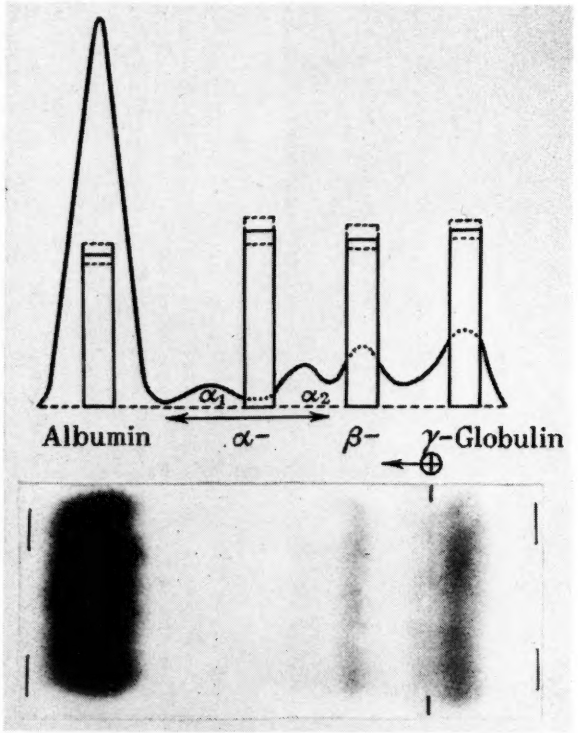

FIG. 1.—An original paperstrip after electrophoresis and staining with bromphenol blue (normal serum No. 3, Table II). Above is the curve as arrived at after quantitative estimation in the Beckman spectrophotometer and the optical density (represented by the height of the columns) of the precipitates of the four fractions.

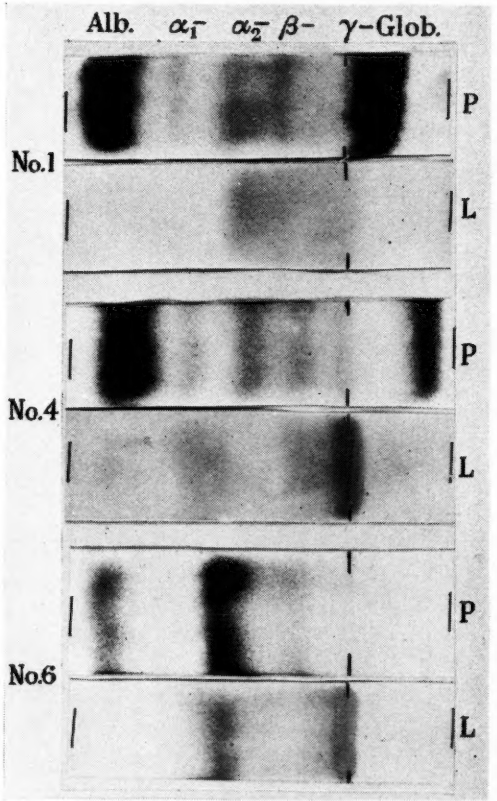

FIG. 2.—Strips of filter-paper after electrophoresis of the sera Nos. 1, 4 and 6 (see Table III). The upper strip of each pair is stained with bromphenol blue and shows the proteins (P); the lower strip is stained with Sudan black/Ciba blue and shows the lipoids (L).

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markable that albumin, α, β and γ globulin, when mixed with an equal volume of antiserum, all showed the optimal zone at dilutions between 1/32 and 1/128 ; thus the precipitations of the main experiments were carried out at the 1/64 dilution of solutions which had identical concentrations of protein to begin with. A volume of 0·5 ml. of the diluted protein was divided into two equal parts and 0·11 ml. of rabbit antibody-serum containing 8 mg. protein was added. Thus the final volume of the reaction mixture was constant, as both the amounts of antiserum and of antigen were the same throughout. The mixture was left standing at room temperature for 3 hr. ; after floccules had formed the tubes were left for two days at 2° and occasionally shaken. This procedure, which yields duplicate results of precipitates at 1/64 dilution, requires 0·9 ml. rabbit antibody-serum for the immuno-chemical titration of the four fractions of a serum. Gitlin (1949) prepares 2 × 7 precipitates per protein (duplicates), which are measured by optical density and are found to be lying on a curve which is characteristic for the tested protein ; we would have followed this procedure had it not required 14 ml. rabbit antiserum per antigen or 56 ml. for the four fractions of a serum. The total volume of rabbit antiserum at our disposal was 26 ml.

The precipitates were washed twice with cold 0·15 M saline and centrifuged at 7500 r.p.m. for 5 min. to recover the specific precipitate. The latter was taken up in 3 ml. 0·1 N NaOH and directly measured in the Beckman spectrophotometer model DU at λ 280 mμ. In accordance with earlier findings (Wunderly and Hässig, 1949) the duplicate results showed a mean deviation of ± 1·9 per cent. Immuno-chemical titrations of two pathological sera were always followed by the titration of the four protein fractions of a normal serum.

RESULTS.

The results are given in the Tables, which show the optical densities of precipitates obtained in triplicate with the fractions of a normal human serum (Table I), and with the fractions of 6 normal sera (Table II) and 9 pathological sera where there was a considerable dysproteinaemia (Table III).

In Table I the specific absorption of the three globulin fractions is very similar because the content of tryptophan, the principal absorbing amino-acid, varies only slightly. The absorption values of albumin are much lower. The optical density of the specific precipitates gives a ratio 1·13 : 1. From column 8 it will be seen that the methodical error of the immuno-chemical titration is about ± 2 per cent. This should be compared with the methodical error of paper electrophoresis (column 9) based on the examination of 25 normal human sera (Grassmann and Hannig, 1952).

Table II shows the optical densities of precipitates of the fractions of six normal human sera. The values are the mean of duplicate analyses (antigen dilution of 1/64).

Fig. 1 shows the stained paper strip of serum No. 3 (Table II) after electrophoresis.

It is of interest to compare immunological titration (Table II, column 9) with electrophoresis. Table II, column 10, gives the result of the examination of 10 normal human sera in the Tiselius apparatus (Sterling, 1949), and column 11 gives the mean deviation of the analyses of 130 sera by paper electrophoresis (Hartmann and Schumacher, 1950). Both these columns differ from column 9, as in electrophoresis the deviation is smaller for the heterogeneous, lipoid-carrying α- and β-globulins. The direct determination of albumin in sera and ascitic fluid by precipitation with rabbit-antiserum showed a mean deviation between ± 3 and ± 5 per cent (Kunkel and Ward, 1950).

Table III presents results obtained with pathological sera. The average optical densities of the precipitates (mean of duplicate analyses) differ little from those obtained with normal sera (Table II), but the mean deviations, as was

TABLE I.—*Optical Densities of Precipitates of the Fractions of a Normal Serum in Triplicate.*

	Protein concentration (mg. per cent).	Optical density at 280 mμ.	Optical density of the precipitate at 280 mμ.			Average	Mean deviation (methodical error) (per cent).	
							Immunochemical titration (per cent).	Paper electrophoresis (per cent).
Albumin	6·33	0·047	0·090	0·092	0·095	0·092	±1·9	±1·2
Globulin α	6·30	0·080	0·102	0·096	0·097	0·099	±2·0	±0·7
Globulin β	6·21	0·078	0·101	0·102	0·100	0·100	±1·7	±0·7
Globulin γ	6·32	0·080	0·104	0·101	0·106	0·104	±1·6	±1·2

TABLE II.—*Comparison of the Optical Densities of Precipitates of the Fractions of Six Normal Sera.*

Serum No.	1.	2.	3.	4.	5.	6.	Average.	Mean deviation (per cent).		
								Immunological titration.	Electrophoresis.	
									(Tiselius).	(Paper).
Total serum protein (g. per cent.	6·8	7·1	7·0	6·9	7·3	6·7				
Albumin	0·090	0·085	0·093	0·091	0·088	0·098	0·0910	±3·2	±2·8	±6·5
Globulin α	0·102	0·116	0·109	0·098	0·107	0·112	0·1073	±5·0	±1·5	±2·5
Globulin β	0·101	0·099	0·106	0·093	0·097	0·107	0·1005	±4·2	±1·2	±3·4
Globulin γ	0·099	0·105	0·109	0·096	0·105	0·106	0·1033	±4·0	±1·7	±5·2

TABLE III.—*Optical Densities of the Precipitates of Serum Protein Fractions with Rabbit Antiserum.*

No.	Sex and age.	Diagnosis.	Cadmium reaction.	Total serum protein (g. per cent).	Serum proteins (percentages of total).					Optical density of precipitates.			
					Albumin.	Globulin.				Albumin.	Globulin.		
						α		β.	γ.		α.	β.	γ.
						α_1.	α_2.						
1	Female 66	Myeloma, γ-globulin type	+++	10·9	12·6	3·0	7·5	6·8	69·9	0·099	0·109	0·107	0·104
2	Male 68	Biliary cirrhosis	+	6·6	49·1	1·6	6·6	8·2	34·5	0·084	0·097	0·095	0·098
3	„ 55	Pulmonary tuberculosis	—	7·2	33·3	7·6	17·9	17·9	23·0	0·097	0·106	0·094	0·096
4	Female 69	Hepatic coma	+	7·0	38·0	5·9	8·1	9·2	38·8	0·094	0·116	0·109	0·109
5	„ 52	Acute yellow atrophy of liver	+++	9·5	15·0	2·1	3·6	5·0	74·3	0·090	0·115	0·106	0·112
6	Male 9	Lipoid nephrosis	—	4·9	15·5	2·7	62·0	10·9	8·9	0·087	0·094	0·091	0·095
7	„ 46	Myeloma, β-globulin type	+++	7·7	17·3	8·2	6·0	42·0	26·6	0·089	0·103	0·109	0·099
8	Female 34	Hepatic coma	—	6·2	51·3	4·0	5·2	10·3	29·2	0·084	0·103	0·090	0·098
9	Male 32	Sprue	—	5·8	55·7	3·9	5·1	17·6	17·6	0·095	0·113	0·102	0·101
					Average :					0·091	0·106	0·100	0·101
					Mean deviations (per cent) :					±4·7,	±6·2,	±7·0,	±4·6.

to be expected, are higher. The greatest differences were seen with the β-globulin, where it is probable that varying amounts of lipoids interfere with precipitation. Lipoids can shield groups which otherwise develop specific activity, but they can also contribute to a decrease in hydrophilic properties of the antigen. It is not possible at this stage to state which tendency prevails. A comparison of the data in columns 10–13 of Table III shows that if a serum has an optical density well above the average all the fractions show high values (see sera 1, 4, 5, 9). Similarly sera with low optical densities show a corresponding uniformity. It is probable that such results could only be obtained with well-separated fractions where specific and non-specific interactions are excluded.

Non-protein-substances in serum.

When working with isolated fractions the most probable reason for a different affinity to the specific antibody are substances of non-protein character like lipoids and mucoids. The difference in the lipoid content of the lipoid-carrying globulin fractions can be well exemplified by staining them with a Sudan black/Ciba blue mixture after paper electrophoresis. To this end two thin lines of the serum with 0·03 ml. each were placed on the same paper. They ran, with 135 V. and 7–8 mA, under exactly the same conditions. After 9 hr. electrophoresis the paper was dried and cut in two. One half was stained with bromphenol blue to show the protein, and the other half was stained with a mixture of Ciba blue and Sudan black to demonstrate the lipoids. Observations so made are presented in Fig. 2 and discussed in the section which follows.

DISCUSSION.

The principal interest of the present paper lies in a comparison of the data in Tables II and III. In the pathological sera the mean deviation of the optical density is found higher in all the protein fractions, and the problem arises whether this increase is sufficiently significant to prove that the fractions of abnormal sera differ immuno-chemically from the normal. The variation in the composition of the sera in Table III is considerable, and the strength of interaction of the different fractions must vary accordingly. One has to expect therefore that a precipitation of unseparated sera with an anti-β-globulin serum or an anti-γ-globulin serum would result in even greater differences. The deviations as we have found them are relatively small because such interactions are ruled out by the technique employed.

The staining of lipoids is suitable as a screening method for selecting material for a detailed and more time-consuming analysis of the various lipoid components. From Fig. 2 it can be seen that the affinity of the different globulin subfractions for lipoids varies. It is well known that no other substance in the serum can occur in such varying quantity as the lipoids. In cases of disseminated lupus erythematosus, in dermatomyositis, in scleroderma and rheumatoid arthritis phospho-lipoids can triple their concentrations, whereas the increase of fatty acids and neutral fat can be tenfold or more. Phospho-lipoids, combining polar and apolar groups in their molecule, have a solubilising effect on the serum proteins. On the other hand, fatty acids and neutral fats decrease the solubility of a serum and make it turbid. Under physiological conditions the α-lipoproteins represent about 3 per cent of the total plasma proteins and

contain about 35 per cent lipoid; for the β-lipoprotein these estimates are 5 per cent and 75 per cent. It is known that the muco-protein and polysaccharide levels of human serum rise sharply in malignant disease as well as in tuberculosis and pneumonia. Normal serum albumin contains 0·61–0·82 per cent polysaccharide and normal α-globulin 1·89–4·00 per cent. Mucoprotein and polysaccharides have the tendency to increase protein solubility. Thus they can contribute, as well as phospho-lipoids, to the prevention of maximum precipitation. A similar effect was found by Krueger and Heidelberger (1951) with water-soluble globulin fractions. Whether globulin subfractions of pathological sera are altered owing to changes in the protein itself cannot be excluded until their amino-acid composition has been measured. From changes in their structure it would be comparatively easy to deduce different hydrophilic or lipophilic properties. Our data suggest that an influence on the physico-chemical properties of globulin fractions is exerted by substances of non-protein character.

SUMMARY.

Serum protein fractions were isolated by paper electrophoresis. The fractions were brought to the same concentration and mixed with rabbit antiserum. The precipitates were measured quantitatively. A comparison was made of the precipitates formed by the fractions of 6 normal and 9 pathological sera. The deviations of values were somewhat greater in pathological than in normal sera, but not sufficiently so to justify an assumption of the presence of atypical proteins. The influence of substances of non-protein character on the size of precipitates is discussed.

REFERENCES.

Albanese, A., Davis, V., Smetak, E., and Lein, M.—(1949) *J. Lab. clin. Med.*, **34**, 326.
Alving, A., and Mirsky, A.—(1936) *J. clin. Invest.*, **15**, 215.
Beaven, C., and Holiday, E.—(1950) *Disc. Faraday Soc.*, **9**, 492.
Chow, B. F.—(1947) *J. biol. Chem.*, **167**, 757.
Deutsch, H. F., Alberty, R. A., Gosting, L. J., and Williams, J. W.—(1947) *J. Immunol.*, **56**, 183.
Eisen, H.—(1948) *Ibid.*, **60**, 77.
Gitlin, D.—(1949) *Ibid.*, **62**, 437.
Idem and Janeway, C.—(1952) *J. clin. Invest.*, **31**, 223.
Goettsch, E., and Reeves, E. B.—(1936) *Ibid.*, **15**, 173.
Grassmann, W., and Hannig, K.—(1952) *Hoppe-Seyl. Z.*, **290**, 1.
Hartmann, F., and Schumacher, G.—(1950) *Z. Naturf.*, **5**^{b}, 361.
Hugentobler, F., Wunderly, C., and Schneider, G.—(1951) *Klin. Wschr.*, **29**, 705.
Jager, B., Smith, E., Nickerson, M., and Brown, D.—(1948) *J. biol. Chem.*, **176**, 1177.
Krueger, R., and Heidelberger, M.—(1951) *Arch. Biochem.*, **34**, 135.
Kunkel, H., and Tiselius, A.—(1951) *J. gen. Physiol.*, **35**, 89.
Idem and Ward, S.—(1950) *J. biol. Chem.*, **182**, 597.
Marrack, J. R., Hoch, H., and Johns, R.—(1951) *Brit. J. exp. Path.*, **32**, 212.
Moore, S., and Stein, W.—(1948) *J. biol. Chem.*, **176**, 367.
Sterling, K.—(1949) *J. clin. Invest.*, **28**, 1057.
Wunderly, C., and Hässig, A.—(1949) *Helv. chim. Acta*, **32**, 1554.
Idem and Reynaud, J.—(1952) *Rev. Hémat.*, **7**, 347.
Idem and Wuhrmann, F.—(1947) *Brit. J. exp. Path.*, **28**, 286.

EFFECT OF DICOUMAROL ON THE ASCORBIC ACID CONTENT OF THE LIVER AND ADRENAL GLANDS.

N. SAPEIKA.

From the Department of Physiology and Pharmacology, University of Cape Town.

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DICOUMAROL has been widely used in man as an anticoagulant. The main action of the drug on the clotting mechanism is generally accepted to be lengthening of the prothrombin time. The drug also diminishes the adhesiveness of platelets and prolongs clot-retraction time ; bleeding time may be prolonged, but capillary fragility is not increased (Thorsén, 1947 ; Jubelirer and Glueck, 1949). Overdosage with the drug leads to haemorrhages into various organs and tissues, which in man have been observed as early as one day and as late as nine months after administration of the drug.

In rats and other laboratory animals toxic doses produce haemorrhages and pulmonary oedema, and centrilobular necrosis of the liver, occurring in rats in about one-half the animals examined, less frequently in mice, dogs and rabbits (Rose, Harris and Chen, 1942). Death can be produced in animals within a few hours (Rose *et al.*, 1942 ; Wakim, Chen and Gatch, 1943). After large doses dilatation of blood vessels and degenerative lesions of their walls have been observed in dogs (Bingham, Meyer and Pohle, 1941 ; McCarter, Bingham and Meyer, 1944), and in dogs and rats (Wakim *et al.*, 1943), although no such changes were observed in dogs or human beings given therapeutic doses. Link (1945) has suggested that dicoumarol in addition to impairing blood coagulation may produce vascular damage.

There are certain gross similarities between the effects of dicoumarol overdosage or of vitamin K deficiency and the haemorrhagic manifestations caused by dietary deficiency of ascorbic acid. The bleeding tendency characteristic of dicoumarol poisoning was suggested by Overman, Stahmann and Link (1942) to be due in part to a disturbance in ascorbic acid metabolism ; they found that ascorbic acid potentiates the anti-dicoumarol action of vitamin K. Although capillary fragility is stated not to be increased by dicoumarol, a decrease in ascorbic acid in the tissues produced by the drug might account for *vascular* changes and haemorrhagic phenomena following overdosage, since deficiency of the vitamin leads to diminution in the strength of the capillaries and widespread haemorrhages.

In view of the similar haemorrhagic manifestations of dicoumarol poisoning and ascorbic acid deficiency, and the possible production of ascorbic acid deficiency by dicoumarol overdosage, it was of interest and practical importance to determine the effect of the drug on the ascorbic acid content of the liver and the adrenal

glands in experimental animals. The investigation was also of interest because some other drugs which damage the rat liver decrease the concentration of ascorbic acid in this organ (Sapeika, 1952).

METHODS.

Since rats synthesise ascorbic acid and do not develop scurvy, guinea-pigs were also used in the present investigation as they are unable to synthesise this vitamin, resemble man in their need for supplies of it, and readily become deficient.

Rats.—Male and non-pregnant female albino rats of the Wistar strain weighing 100–180 g. were used. They were fed powdered stock diet (Thomson, 1936) containing dicoumarol in the following concentrations: 1·0, 0·5, 0·1, 0·05, 0·01 g. per 100 g. diet. Control animals received powdered stock diet only. No green food was given during the experiments. The concentrations of dicoumarol used are high; they were the same as those administered by Rose *et al.* (1942) in their studies on the toxicity of dicoumarol.

Guinea-pigs.—Male and non-pregnant females weighing 260–360 g. were used. They were divided into groups of 16. Group 1 was given the rat stock diet (Thomson, 1936), which is scorbutic for guinea-pigs, though not for rats, which can synthesise ascorbic acid. Group 2 was given stock diet, and ascorbic acid 2·5 mg. in 0·25 ml. water was administered by mouth to each animal daily. Group 3 received stock diet containing dicoumarol 0·05 per cent. Group 4 received the same mixture as Group 3, but ascorbic acid 2·5 mg. in 0·25 ml. water was given by mouth to each animal every morning.

Rats and guinea-pigs from each group were killed (by a blow on the head and bleeding from incision of the neck vessels) at varying intervals after the beginning of the experiments. Freshly excised liver tissue and the adrenal glands were immediately assayed for their ascorbic acid content by the method of Kennaway, Kennaway and Warren (1944) and Kennaway and Tipler (1947) as in the previous investigation (Sapeika, 1952). The amounts of ascorbic acid are expressed as mg./100 g. fresh tissue. Control values for rat liver ascorbic acid ranged from 22·5 to 31 (mean 26), for the adrenal glands 384 to 560 (mean 480), and for guinea-pig tissues as shown in Table III.

RESULTS.

Rats.—The effect of dicoumarol on the ascorbic acid content of liver and adrenal glands is shown in Table I. A few animals in each group died during

TABLE I.—*Ascorbic Acid Content of Liver and Adrenals in Rats Treated with Dicoumarol (mg./100 g. fresh tissue).*

Days of feeding.	Amount of dicoumarol in diet (per cent). 1·0. Liver.	1·0. Adr.	0·5. Liver.	0·5. Adr.	0·1. Liver.	0·1. Adr.	0·05. Liver.	0·05. Adr.	0·01. Liver.	0·01. Adr.
1	22 (2)	277	27 (4)	420						
2	17 (2)	240	22 (4)	378						
3	16 (8)	204	17 (4)	320	25 (4)	520				
4	16 (3)	177	—		27 (6)	445	26 (2)	420		
5	—		15 (2)	300	—		26 (2)	305	31 (4)	484
6	—		14 (4)	315	20 (13)	329	23 (4)	338		
7	—		11 (5)	182	21 (6)	313	22 (4)	315		
8	—		9 (2)	170	19 (10)	328	20 (3)	300		
9	—		—		—		—		27 (4)	432
14	—		—		—		—		28 (4)	505
19	—		—		—		—		29 (4)	496

The number of rats used for estimations on each day is indicated in brackets.

the experiments and were not used for estimations. Others became weak and at autopsy might be found to have internal haemorrhages with sometimes a pale

liver. Most animals survived several days after a decrease in ascorbic acid had become evident before showing evidence of the toxic effects of dicoumarol. Most animals survived not more than 9 days when receiving dicoumarol 1, 0·5, 0·1, and 0·05 per cent in the diet, as was found by Rose *et al.* (1942). Examinations were made on all surviving members in each group. The earlier deaths of some animals indicate the variability of response to dicoumarol poisoning even in normal animals.

In a further experiment a group of rats received dicoumarol 0·05 per cent in stock diet, and ascorbic acid 10 mg. in 0·25 ml. water was administered daily by stomach-tube. The ascorbic acid in liver and adrenals was maintained at high normal levels (see Table II), but did not prevent the appearance of haemorrhages from about the fourth day after the start of the experiment.

TABLE II.—*Effect of Dicoumarol* 0·05 *per cent plus Ascorbic Acid* 10 *mg. Daily in the Rat.*

Days.	Number of animals.	Ascorbic acid (mg. per 100 g.).	
		Liver.	Adrenals.
4	4	32	430
6	4	24	417
7	4	27	488
8	4	27	432
10	4	26	491

Rats given a single large dose of dicoumarol, 300 mg. per kg. (suspended in water with compound of tragacanth, and administered through a stomach-tube), showed no significant decrease in liver and adrenal ascorbic acid at 6, 24 or 48 hours after administration of the drug.

Guinea-pigs.—The results are summarised in Table III. Four animals (2 male, 2 female) were used on the day stated for ascorbic acid estimations. In Groups

TABLE III.—*Ascorbic Acid Content of Liver and Adrenals in Guinea-pigs (mg./100 g. fresh tissue).*

Days of feeding.	Group 1: Stock diet (scorbutic).		Group 2: Stock diet + ascorbic acid.		Group 3: Stock diet + dicoumarol.		Group 4: Stock diet + dicoumarol and ascorbic acid.	
	Liver.	Adrenals.	Liver.	Adrenals.	Liver.	Adrenals.	Liver.	Adrenals.
4	4·5	33	9·4	53	5·4	36	10	55
5	4·25	33	12	56	5	27	11	58
6	4	22	9	43	4·5	26	12	46
	(No haemorrhages)				(Severe		(Severe	
14	3·25 (Haemorrhages)	7·5	5·5 (No haemorrhages)	45	haemorrhages in all animals)		haemorrhages in all animals)	

3 and 4 haemorrhages occurred in all animals, sometimes very gross, in some instances fatal; these were intra-abdominal, intramuscular or subcutaneous. None of the animals in Groups 1 and 2 showed haemorrhages and none died during the corresponding period of six days; at 14 days, however, all animals in Group 1 but none in Group 2 (controls) showed scorbutic haemorrhages, especially around the knees, but no large haemorrhages elsewhere, such as were found in the dicoumarol-poisoned animals.

Thus in Groups 1 and 3 the ascorbic acid levels are low, but only the dicoumarol-treated animals developed haemorrhages or died. In Groups 2 and 4 the ascorbic acid levels are high, yet only the dicoumarol-treated animals developed haemorrhages or died ; *i.e.*, with these amounts of ascorbic acid in the tissues the animals were not protected.

In a further experiment two groups of guinea-pigs which received dicoumarol 0·01 per cent in stock diet and daily oral administration of ascorbic acid 2·5 mg. and 10 mg. respectively showed high normal levels of ascorbic acid in liver and adrenal glands (Table IV), yet the development of large haemorrhages and some deaths from the fourth day of the experiment was not prevented.

TABLE IV.—*Effect of Dicoumarol + Ascorbic Acid in Guinea-pigs.*

Days.	Number of animals.	Stock diet : Dicoumarol 0·01 per cent, ascorbic acid 2·5 mg. daily.		Number of animals.	Stock diet : Dicoumarol 0·01 per cent, ascorbic acid 10 mg. daily.	
		Liver.	Adrenals.		Liver.	Adrenals.
4	4	8·25	68	4	11	86
6	4	7·75	57	2	12	80
9	2	7·5	55	2	10·5	80

DISCUSSION.

The investigation shows that dicoumarol given to rats in doses much larger than those used for therapeutic purposes in man can cause a progressive decrease in the ascorbic acid content of the liver and the adrenal glands. With 1 per cent of the drug in the food the decrease is first noticeable in about one day, with 0·5 per cent in about two days, with 0·1 per cent in four to six days, with 0·05 per cent in five to six days from the commencement of feeding ; with 0·01 per cent no decrease was observed over a period of nineteen days.

It is well known that rats synthesise ascorbic acid, presumably in the intestine (Svirbely, 1936), and diminished intake of food or a scorbutic diet does not produce any remarkable decrease in the vitamin content of the animal ; on the other hand certain hepatotoxic drugs (Sapeika, 1952) and a yeast diet necrogenic to the liver (Lindan and Work, 1951) produce a marked decrease in liver ascorbic acid. The mode of action of dicoumarol in producing the decrease in ascorbic acid may possibly be related to the toxic action of the drug on the liver and other tissues, or to interference with the synthesis or to increased excretion of this vitamin. Dicoumarol does not appear to act like the closely related sodium salicylate, which in a single large dose given subcutaneously rapidly depletes adrenal ascorbic acid (Cronheim, King and Hyder, 1952*a*, *b*) ; a single large dose of dicoumarol, 300 mg. per kg., given orally to rats was not found to decrease the ascorbic acid levels in the present investigation, and one or three doses of 25 mg. each given orally to rats was found by Baumann, Field, Overman and Link (1942) to produce an increase in the concentration of ascorbic acid in the blood but not in other tissues.

The initial experiments of the present investigation on rats suggested that in man certain of the haemorrhagic features of dicoumarol overdosage, which resemble the features of classical scurvy, might result from a rapid decrease in ascorbic acid, especially in patients receiving dicoumarol who are already in a

poor nutritional state; with smaller doses of dicoumarol the more commonly encountered type of hypovitaminosis or subscorbutic state might be produced rather than the full picture of scurvy. The fact that the anticoagulant action of dicoumarol requires large doses of synthetic vitamin K to counteract the action and that the closest study of prothrombin concentrations does not always indicate when haemorrhages will occur shows that dicoumarol has other effects on the coagulation mechanism. Bollman and Preston (1942) suggested that some other factor than depression of prothrombin seems necessary for the development of haemorrhage. In the present investigation rats and guinea-pigs receiving ascorbic acid with dicoumarol still developed haemorrhages. Baumann *et al.* (1942) also found that in rats the administration of ascorbic acid failed to alter the coagulation defect induced by dicoumarol and failed to prolong the lives of the animals. On the other hand, in studies on a small number of guinea-pigs, Richards and Cortell (1942) found a tendency of vitamin C-depleted animals to succumb earlier to the dicoumarol; they considered there might be a connection between vitamin C level and sensitivity to dicoumarol. The relative doses of dicoumarol and ascorbic acid may be important.

When bleeding occurs in a patient receiving dicoumarol it is generally recommended that transfusions of fresh blood or plasma, or large doses of vitamin K, should be administered. Ascorbic acid would not appear to be of value in the prevention or treatment of dicoumarol haemorrhages, but investigations on man are needed to clarify this point. Estimations of the ascorbic acid content of the plasma or preferably of the white cell-platelet layer of centrifuged blood by more sensitive methods than those used in this study, and the effect of ascorbic acid administration in patients receiving dicoumarol, especially over a prolonged period, might reveal a relationship between the experimental findings and the action of dicoumarol on ascorbic acid metabolism in man.

SUMMARY.

The haemorrhages resulting from dicoumarol overdosage have not been fully explained.

In rats the administration of dicoumarol 0·05 per cent or more in the diet produces a decrease in the ascorbic acid content of the liver and the adrenal glands. Although death or haemorrhage occurs relatively early in a few animals, most survive several days, and may show no gross haemorrhage even when the vitamin levels are already low. Haemorrhages produced by dicoumarol are not prevented by giving ascorbic acid.

In guinea-pigs dicoumarol 0·01 per cent in the diet rapidly produces severe haemorrhages and death within a few days, yet control animals with the same levels of ascorbic acid do not develop haemorrhages and survive, unless inadequate dietary intake of the vitamin produces scurvy many days later. Animals receiving supplementary doses of ascorbic acid are not protected against the toxic effects of dicoumarol.

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REFERENCES.

BAUMANN, C. A., FIELD, J. B., OVERMAN, R. S., AND LINK, K. P.—(1942) *J. biol. Chem.*, **146**, 7.

BINGHAM, J. B., MEYER, O. O., AND POHLE, F. J.—(1941) *Amer. J. med. Sci.*, **202**, 563.

BOLLMAN, J. L., AND PRESTON, W.—(1942) *J. Amer. med. Ass.*, **120**, 1021.

CRONHEIM, G., KING, J. S., AND HYDER, N.—(1952*a*) *Fed. Proc.*, **11**, 334.—(1952*b*) *Proc. Soc. exp. Biol., N.Y.*, **80**, 51.

JUBELIRER, R. A., AND GLUECK, H. I.—(1949) *J. Lab. clin. Med.*, **34**, 448.

KENNAWAY, E. L., KENNAWAY, N. M., AND WARREN, F. L.—(1944) *Cancer Res.*, **4**, 245.

Idem AND TIPLER, M. M.—(1947) *Brit. J. exp. Path.*, **28**, 351.

LINDAN, O., AND WORK, E.—(1951) *Biochem. J.*, **48**, xxxi.

LINK, K. P.—(1945) *Harvey Lect.*, **39**, 162.

MCCARTER, J. C., BINGHAM, J. B., AND MEYER, O. O.—(1944) *Amer. J. Path.*, **20**, 651.

OVERMAN, R. S., STAHMANN, M. A., AND LINK, K. P.—(1942) *J. biol. Chem.*, **145**, 155.

RICHARDS, R. K., AND CORTELL, R.—(1942) *Proc. Soc. exp. Biol., N.Y.*, **50**, 237.

ROSE, C. L., HARRIS, P. N., AND CHEN, K. K.—(1942) *Ibid.*, **50**, 228.

SAPEIKA, N.—(1952) *Brit. J. exp. Path.*, **33**, 223.

SVIRBELY, J. L.—(1936) *Amer. J. Physiol.*, **116**, 446.

THOMSON, W.—(1936) *J. Hyg., Camb.*, **36**, 24.

THORSÉN, G.—(1947) *Lancet*, ii, 420.

WAKIM, K. G., CHEN, K. K., AND GATCH, W. D.—(1943) *Surg. Gynec. Obstet.*, **76**, 323.

THE INHIBITION OF THE HAEMAGGLUTININS IN PLANT SEEDS BY HUMAN BLOOD GROUP SUBSTANCES AND SIMPLE SUGARS.

W. T. J. MORGAN AND WINIFRED M. WATKINS.

From the Lister Institute of Preventive Medicine, London, S.W.1.

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THE agglutination of human red cells by saline extracts of plant seeds has been examined in considerable detail (Renkonen, 1948, 1950; Koulumies, 1949; Boyd and Reguera, 1949; Boyd, 1950; Krüpe, 1950; Krüpe and Braun, 1952; Cazal and Lalaurie, 1952; Bird, 1952). In an attempt to understand more fully the difference in the specific serological characters of these red cell agglutinins a study has been made of the reactivity of extracts prepared from *Lotus tetragonolobus*, *Sophora japonica*, *Laburnum alpinum*, *Vicia cracca*, Lima beans and *Cytisus sessilifolius* with cells of known phenotype, and of their inhibition by preparations of the human blood group substances and of simple sugars of known constitution. The results of these experiments are given in this paper.

MATERIALS AND METHODS.

The seed extracts were made by soaking the finely ground seed material in ten times its weight of saline for 2 hr. at 37°. The insoluble seed residues were then removed by centrifugation and the clear supernatant fluid used for agglutination tests. An amount of agglutinin for the selected erythrocytes equivalent to 2–3 completely agglutinating doses was used in all inhibition tests.

The seed extract was titrated against a panel of freshly collected human red cells of known phenotype. The same donors were used throughout the investigation. Agglutination and inhibition tests were read after standing for 2 hr. at 16–20°.

The preparation and properties of the human blood group substances used in the inhibition tests have already been described (Aminoff, Morgan and Watkins, 1950; Annison and Morgan, 1952*a*, *b*; Gibbons and Morgan, 1952). Jelly coat substance obtained from the egg of the sea urchin was kindly supplied by Dr. E. Vasseur of the Wenner-Grens Institute, Stockholm. The O somatic antigen of *Shigella shigae* was prepared as described earlier (Morgan, 1937). The specific substance of *Trichomonas foetus* var. Manley was as prepared by Feinberg and Morgan (1953).

RESULTS.

Lotus tetragonolobus *extract.*

The haemagglutinins contained in the extract reacted with the test cells as shown in Table I. The results show, in agreement with the earlier findings of Renkonen (1948), that group O and A_2 cells are agglutinated to a higher titre than are the other cells, and therefore the reagent can be used after suitable dilution as an O cell agglutinin.

The results given in Table II show that a test dose of the extract, which is without action on A_1, B and A_1B cells, is completely inhibited in its action on

TABLE I.—*The Agglutination of Human Erythrocytes of Different Phenotype by Aqueous Extracts of Various Plant Seeds.*

Blood group of test cells.	Agglutination end-point (2 hr. 18°) with 10 per cent aqueous extract of—					
	Lotus tetragonolobus.	*Sophora japonica.*	*Vicia cracca.*	Lima bean.	*Laburnum alpinum.*	*Cytisus sessilifolius.*
A_1	1 : 16	1 : 256	1 : 16,384	1 : 512	1 : 8	1 : 8
A_2	1 : 128	1 : 64	1 : 8,192	1 : 128	1 : 32	1 : 32
B	1 : 16	1 : 1,024	1 : 128	1 : 4	1 : 16	1 : 16
O	1 : 256	1 : 16	1 : 64	<1 : 2	1 : 64	1 : 128
A_1B	1 : 2	1 : 512	1 : 8,192	1 : 256	<1 : 2	<1 : 2
A_2B	1 : 32	1 : 256	1 : 1,024	1 : 32	1 : 16	1 : 32

TABLE II.—*The Inhibition by H-substance and Simple Sugars of the Agglutination of Group O Cells by an Aqueous Extract of the Seeds of* Lotus tetragonolobus *and the Anti-H Agglutininin in Natural Eel Serum.*

Inhibiting substance.	Dilution of substance giving inhibition end-point.	
	Lotus tetragonolobus extract.	Eel serum.
Human H-substance	1 : 25,600	1 : 819,200
Hog H-substance	1 : 25,600	1 : 1,638,400
L-Fucose	1 : 25,600	1 : 12,800
α-Methyl-L-fucopyranoside	1 : 51,200	1 : 51,200
β-Methyl-L-fucopyranoside	1 : 12,800	1 : 3,200
2-Deoxy-L-fucose	1 : 12,800	1 : 6,400
L-Galactose	1 : 3,200	<1 : 50
6-Deoxy-L-talose	1 : 400	1 : 800
D-Arabinose	1 : 200	1 : 400
D-Digitoxose	1 : 200	<1 : 50
N-Acetylglucosamine	1 : 200	<1 : 50
D-Ribose	1 : 100	<1 : 50
D-Fructose	<1 : 50	1 : 200
D-Fucose	<1 : 50	<1 : 50

group O cells by an equal volume of a 1 : 25,600 dilution of the purified human H-substance. This is a relatively low inhibiting activity in comparison with that given by the same H-substance when used in inhibition tests employing approximately the same number of agglutinating doses of the more commonly used anti-H reagents, such as natural eel serum (Grubb, 1949), or anti-H sera produced in the rabbit (Morgan and Waddell, 1945 ; Annison and Morgan, 1950). The observation (Watkins and Morgan, 1952) that the anti-H agglutinin in eel serum can be neutralised by L-fucose and other closely related sugars made it of interest to determine whether the agglutination of O cells by the *Lotus tetragonolobus* extract could be inhibited by any of the simple sugars which are components of the H-substance, *i.e.*, L-fucose, D-galactose, N-acetyl-D-glucosamine and N-acetyl-D-chondrosamine (Annison and Morgan, 1952*b*). Preliminary experiments indicated that L-fucose neutralised the seed agglutinin, and since structural studies on the H-substance indicate that at least a part of the fucose contained in the complex is joined to the other components through a glycoside linkage, α and β methyl-L-fucopyranosides were included in the test substances. The results of inhibition tests using these materials and a number of other closely related sugars are given in Table II.

The α-methyl-L-fucopyranoside was more active than the parent sugar, and gave a higher inhibition end-point than either the human or hog H-substances.

The anomer, β-methyl-L-fucopyranoside, and 2-deoxy-L-fucose, in which the hydroxyl group at carbon atom 2 in L-fucose is replaced by hydrogen, were slightly less active than L-fucose. L-galactose, which differs from L-fucose in that it possesses a $-CH_2OH$-group at carbon atom 6 in place of the CH_3 group, showed a further loss in specific activity. 6-Deoxy-L-talose, D-arabinose, D-digitoxose, N-acetylglucosamine, and D-ribose showed slight inhibiting activity only. The glucosaminides, N-acetyl-α-methylglucosaminide, N-benzoyl-methyl glucosaminide and N-acetyl-β-phenylglucosaminide failed to inhibit at the highest concentration (2 per cent) tested.

The following sugars and amino-acids, tested at a concentration of 2 per cent and polysaccharides, tested at 1 per cent concentration, were also without inhibiting activity: D-fucose, D-glucosamine, D-chondrosamine, N-acetylchondrosamine, D-fructose, sucrose, D-galactose, D-mannose, L-sorbose, L-arabinose, L-gulo-methylose, D and L-xylose, D-altrose, D-manno-D-*gala*heptose, D-manno-heptulose, D-gluco-L-*gala*octose, D-glucoheptulose, D-gulo-L-*gala*heptose, D-gluco-L-*talo*-octose, D and L-glucose, L-rhamnose, L-arginine, L-cystine, methionine, serine, valine, lysine, histidine, tyrosine, isoleucine, proline, threonine, norleucine, phenylalanine, hydroxyproline, aspartic acid, fucoidin, gum arabic, gum tragacanth, dextran, Type XIV pneumococcus polysaccharide (0·1 per cent), O somatic antigen of *Shigella shigae*, and jelly coat substance from sea urchin's egg, and the specific substance isolated from *T. foetus*.

The agglutination of O cells by the *Lotus tetragonolobus* extract was not inhibited by purified samples of blood group A, B or Le^a substances. An extract prepared from a group O stroma inhibited at a dilution of 1 : 1,600.

The sugars which neutralised the O cell agglutinin in the *Lotus tetragonolobus* extract were for the main part the same as those which combined with the anti-H agglutinin in eel serum, but some differences were observed in the capacity of certain sugars to inhibit the two reagents. The inhibition end-points obtained with eel serum are included in Table II.

Sophora japonica *extract*.

A 10 per cent aqueous extract of the seeds of *Sophora japonica* agglutinated human red cells as shown in Table I. It will be seen that the agglutinin acts on A and B more strongly than on O cells. The intensity with which purified preparations of the A and B group substances were able to inhibit the agglutination of A_1, A_2 and B cells by this seed extract is given in Table III, and shows, surprisingly, that A substance inhibits the action of *Sophora* extract on B cells and that B substance similarly inhibits the action of the agglutinin on A cells. These results suggest that the extract contains an agglutinin which possesses a receptor common to both the A and B specific configurations; this might be termed C agglutinin, since agglutinins of human and animal origin which possess a capacity to react with either A or B receptors have been recorded (Hooker and Anderson, 1921; Wiener, 1943). The existence of an agglutinin which possesses a receptor specific for a structure common to both the A and B substance is indicated from the results of the following experiment: Two similar amounts of *Sophora* extract were absorbed with either A_1 or B cells at about 5°. The cells were removed at this temperature by centrifugation, washed three times with ice-cold saline and finally re-suspended in a small volume of fresh saline. The cell suspension was then heated at 50°–55° for 10 min. and centrifuged at this temperature. The

TABLE III.—*The Inhibition by A and B Blood Group Substances and* N-*acetylchondrosamine of the Agglutination of Human Red Cells of Different Phenotypes by* Sophora japonica *Extract.*

Substances used for inhibition.	Test cells.	Inhibition end-point.
A-substance	A_1	1 : 12,800
	A_2	1 : 192,000
	B	1 : 12,800
B-substance	A_1	1 : 192,000
	A_2	1 : 192,000
	B	1 : 384,000
N-Acetylchondrosamine	A_1	1 : 1,600
	A_2	1 : 6,400
	B	1 : 1,600

eluate was found to agglutinate both A_1 and B cells and was inhibited in its action on these cells by the A or B specific blood-group substances. If the absorption of the *Sophora* extract was carried out with group O cells in place of A_1 or B cells, the eluate was devoid of agglutinins for A_1 or B cells.

Inhibition tests were made using a saline extract of *Sophora japonica* seeds as agglutinin and simple sugars of known configuration as inhibitors. In view of the reactivity of the agglutinating reagent with both A and B cells, erythrocytes belonging to groups A_1, A_2 and B were included in most tests, and the dilution of the extract used was varied according to the group of the test cells to ensure that approximately the same number of agglutinating doses was employed. Included in the simple sugars tested were L-fucose, D-galactose, N-acetyl-D-glucosamine and N-acetyl-D-chondrosamine, components of the A and B specific substances. The most active inhibitor found in the group of substances examined was N-acetylchondrosamine (Table III). N-Acetylglucosamine, on the other hand, showed no capacity to inhibit the agglutination of either A_1, A_2 or B cells, and the hydrochlorides of glucosamine and chondrosamine likewise failed to inhibit at concentrations less than about 0·5 per cent. D-Galactose and melibiose were somewhat more active (1 : 800 and 1 : 400 respectively) and lactose gave inhibition at a concentration of 1 : 1600 using group B test cells. L-Arabinose and D-fucose showed slight inhibiting activity (1 : 200) with A_1, A_2 and B cells.

Sugars, other than those mentioned above, and amino-acids used in the inhibition tests with the *Lotus tetragonolobus* agglutinin were examined similarly with *Sophora* extract and were found to be without significant activity. Slight activity at a dilution of 1 : 400 was observed with human H-substance and with blood group Le^a substance. A material obtained from O stroma inhibited at a dilution 1 : 1600 and the specific substance from *T. foetus* was active at 1 : 400.

Vicia cracca *extract.*

The behaviour of red cells of different phenotypes with the *Vicia cracca* extract is shown in Table I. A_1 and A_2 cells are agglutinated much more strongly than O.

Purified human blood group A substance inhibited the agglutination of A_1 cells by *Vicia cracca* extract completely at a dilution of 1 : 2,000,000, whereas only one simple sugar, N-acetylchondrosamine, shows appreciable activity (1 : 3,200). The parent sugar, chondrosamine hydrochloride, was active in much higher concentration only (1 : 200), whereas glucosamine required a 1 per cent solution to bring about complete inhibition of agglutination. N-acetylglucosamine was in-

active at a concentration of 2 per cent. The other sugars and amino-acids employed in the *Lotus tetragonolobus* experiments failed to bring about inhibition of the anti-A agglutinin in the *Vicia cracca* extract. Some inhibition was shown by the O somatic antigen of *Shigella shigae*, and by the jelly coat substance from the sea urchin's egg (1 : 800) and with the specific substance of *T. foetus* (var. Manley) (1 : 200). Purified human B substance failed to inhibit at a dilution of 1 : 200, but slight activity was observed with human and hog H-substances (1 : 800) and with Le^a substance (1 : 400). No inhibition was obtained with a 1 : 100 dilution of the material extracted from O stroma.

Lima bean extract.

A 10 per cent saline extract of Lima beans agglutinated A_1 and A_2 cells very much more strongly than it did B and O cells, as shown in Table I.

Purified group A substance inhibited the agglutination of A_1 cells by the Lima bean extract at a dilution of 1 : 500,000, whereas preparations of human B, H and Le^a substances and material extracted from O stroma were without activity at a dilution of 1 : 100. Inhibition tests with the same simple sugars, sugar derivatives and amino-acids as were used in the *Lotus tetragonolobus* experiments gave negative results at a dilution of 1 : 50 with the exception of N-acetylchondrosamine, which again showed some slight capacity to neutralise the Lima bean agglutinin at this concentration.

Laburnum alpinum *and* Cytisus sessilifolius *extracts.*

Extracts in saline (10 per cent) of the seeds of *Laburnum alpinum* and *Cytisus sessilifolius* agglutinated cells of group O and A_2 more strongly than cells of other groups (Table I).

The agglutination of O cells by the two extracts was inhibited by preparations of human group substances and the results are shown in Table IV. None of the

TABLE IV.—*The Inhibition by Blood Group Substances and other Biological Materials of the Agglutination of Group O Cells by Aqueous Extracts of* Laburnum alpinum *and* Cytisus sessilifolius *Seeds.*

Inhibiting substance.	Dilution of substance giving inhibition end-point with aqueous extract of—	
	Laburnum alpinum.	*Cytisus sessilifolius.*
Human H-substance	1 : 1,638,400	1 : 204,800
Hog H-substance	1 : 3,276,800	1 : 819,200
Human A-substance	1 : 6,400	1 : 3,200
Human B-substance	1 : 25,600	1 : 51,200
O stroma extract	1 : 12,800	1 : 6,400
Somatic antigen from *Shigella shigae*	1 : 400	1 : 400
Specific substance from *Trichomonas foetus*	<1 : 50	<1 : 50

simple substances of known structure used in the inhibition tests with the other seed extracts showed any capacity, at a concentration of 2 per cent, to neutralise the O cell agglutinins extracted from either *Laburnum alpinum* or *Cytisus sessilifolius*.

DISCUSSION.

The discovery by Renkonen (1948) that extracts of certain seeds could be used to differentiate the blood-group characters of human erythrocytes provided further evidence for the wide distribution in nature of materials related serologically to

the antigens and antibodies classified within the human ABO blood group system. Substances which neutralise the human iso-agglutinins, for example, are found in the secretions and tissues of many mammals and certain bacteria (Landsteiner, 1947), and agglutinins which react preferentially with red cells belonging to one of the ABO groups are encountered in many animal sera (Wiener, 1943; Race and Sanger, 1950), and more recently have been reported in the sera of fish (Cushing, 1952) and extracts of fungi (Elo, Estola and Malmström, 1951). The work presented in this paper was undertaken in an attempt to learn more of the speci-

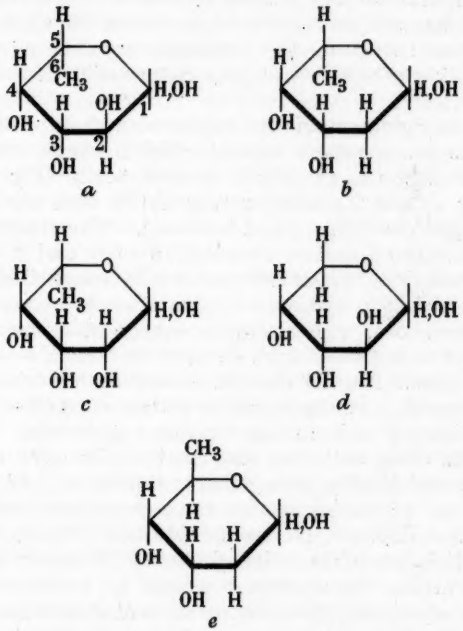

FIG.—Structural formulae of *a*, L-fucose, ; *b*, 2-deoxy-L-fucose ; *c*, 6-deoxy-L-talose ; *d*, D-arabinose ; *e*, D-digitoxose.

ficity of certain of the plant seed haemagglutinins by an examination of their behaviour in inhibition tests with the purified blood group substances, other materials of biological interest and simple compounds of known structure.

Aqueous extracts of the seeds of three plants, *Lotus tetragonolobus*, *Laburnum alpinum*, and *Cytisus sessilifolius*, which have been shown (Renkonen, 1948) to react preferentially with cells of group O were examined. The results obtained with the haemagglutinins present in seeds of *Lotus tetragonolobus* were of most interest, because with this extract certain simple sugars were found to give inhibition. The highest inhibition end-point was obtained with α-methyl-L-fucopyranoside and, indeed, this compound was found to be more active under the test conditions than were the purified H-substances of human or animal origin. In this respect the results differed to some extent from those obtained with the

anti-O cell agglutinin in eel serum since, although α-methyl-L-fucoside was also the most active of the simple sugars tested in inhibiting the eel agglutinin, the inhibiting activity was equivalent to only a few per cent of the activity of the human H-substance. Other differences observed were that L-galactose, which differs from L-fucose (Fig. *a*) only in the replacement of the CH_3 group at carbon atom 6 by a CH_2OH group, showed no capacity to inhibit the eel serum agglutinin, whereas appreciable inhibition of the *Lotus tetragonolobus* agglutinin was obtained with this sugar. The *Lotus* agglutinin was also inhibited weakly by D-digitoxose and D-ribose which had no activity against eel anti-H serum, and was not inhibited by D-fructose which showed some power to neutralise the agglutinin in eel serum. The specificity of these two antibodies, although very closely related, is not therefore identical. In view of the heterologous nature of both these agglutinins this finding is not surprising.

Apart from N-acetylglucosamine the sugars which inhibited the agglutination of O cells by *Lotus tetragonolobus* extract differ in configuration from L-fucose (Fig. *a*) at a single carbon atom only [2-deoxy-L-fucose (Fig. *b*), L-galactose, 6-deoxy-L-talose (Fig. *c*) and D-arabinose (Fig. *d*)] or with weaker activity at two carbon atoms [D-digitoxose (Fig. *e*) and D-ribose]. The changes in configuration involve the substituents at carbon atoms 2, 5 or 6, and it is of particular interest that L-gulomethylose, which differs in configuration from L-fucose only by the reversal of the hydrogen-hydroxyl configuration at carbon atom 3, failed to inhibit. L-Glucomethylose, with a similar change at carbon atom 4, was not available for test, but L-glucose with changes at carbon atom 4 and 6, and L-rhamnose with changes at 2 and 4, showed no activity ; it would appear probable therefore that differences in configuration at carbon atoms 3 or 4 are incompatible with retention of capacity to neutralise the *Lotus* agglutinin.

The relative inhibiting activities discussed so far have been considered in terms of the maximum dilution giving rise to inhibition. If, however, the inhibition end-points are expressed as molar concentrations instead of as simple dilutions a somewhat different picture is obtained. Thus, H-substance most probably has a particle size of the order of 300,000 (Kekwick, 1952 ; Annison and Morgan, 1952*b*), whereas the molecular weight of α-methyl-L-fucoside is 178. Expressed in terms of molarity the concentration of H-substance at the inhibition end-point with *Lotus* agglutinin is $1{\cdot}3 \times 10^{-4}$ mM, whereas the corresponding concentration of the fucoside is $1{\cdot}1 \times 10^{-1}$ mM. It is evident therefore that although the latter sugar appears to be more active when the concentration is expressed as a simple dilution, the number of molecules of the fucoside required to neutralise completely the agglutinin is in fact approximately 1000 times as great as the number of molecules of the H-substance. The fucose content of the H-substance used in this investigation is about 14 per cent, and it may be calculated that there are approximately 250 fucose residues per molecule. If all these fucose residues are free to react with the *Lotus* agglutinin, each particle of H-substance has theoretically 250 times as many combining sites as each molecule of α-methyl-L-fucopyranoside. A concentration of the fucoside of $1{\cdot}1 \times 10^{-1}$ mM is equivalent, therefore, to about four times as many specific groupings as a $1{\cdot}3 \times 10^{-4}$ mM concentration of H-substance. It is improbable that the whole of the fucose in the H-substance is bound structurally in the same way, and it is unlikely therefore that all the fucose residues are available for combination with the antibody. Furthermore, even though the H-substance has some 250 fucose

residues in its molecule, it is improbable that it would be able to combine with anything approaching this number of agglutinin molecules owing to steric factors. On the basis of these calculations it would appear that the H-substance is more closely complementary to the *Lotus* agglutinin than is the α-methyl-L-fucopyranoside, but it may well be an α-fucoside grouping which is largely responsible for the reactivity of the agglutinin with O cells.

The agglutination of O cells by the extracts obtained from *Laburnum alpinum* and *Cytisus sessilifolius* seeds was neutralised by human and hog H-substances; these seed agglutinins could therefore be classified, together with the *Lotus tetragonolobus* extract and the eel serum agglutinin, as having anti-H specificity. It is to be noted, however, that whereas the *Lotus* extract and eel serum agglutinin were inhibited by α-methyl-L-fucopyranoside, neither this substance nor any of the sugars or amino-acids examined showed any capacity to neutralise the anti-H agglutinins present in the *Laburnum alpinum* or *Cytisus sessilifolius* extracts. In this respect the latter two agglutinins behaved similarly to the other anti-H sera of human and animal origin examined previously for inhibition with simple substances of known structure (Watkins and Morgan, 1952).

Marrack and Carpenter (1938) measured the inhibition by simple haptens of precipitate formation between Type II pneumococcus antiserum and various gums, and suggested that the amount of a given simple hapten required to inhibit precipitate formation may be considered as a measure of the relative affinities of antibody for antigen and simple hapten. If this argument is applied to the inhibition given by human H-substance with *Lotus tetragonolobus*, *Laburnum alpinum* and *Cytisus sessilifolius*, it is evident that the agglutinin in the *Lotus* extract has less affinity for the H-substance than have the *Laburnum alpinum* and *Cytisus sessilifolius* extracts. The homologous antigens or haptens for the seed agglutinins are unknown, but the amount of extract used in the inhibition tests with all three agglutinins was adjusted to contain approximately equivalent amounts of antibody for the heterologous receptor on the red cell surface, and the results therefore suggest that the receptor with which the *Laburnum* and *Cytisus* agglutinins are reacting is much more closely related to the isolated and purified H-substance than is the receptor detected by the *Lotus* agglutinin.

Powerful anti-A agglutinins have been discovered in extracts of *Vicia cracca* and Lima beans (Renkonen, 1948; Boyd and Reguera, 1949). Examination of the behaviour of these two seed extracts in inhibition tests showed that the agglutination of A_1 cells was inhibited to high titre by purified human A substance, and that human B substance was without activity. N-Acetylchondrosamine alone of the simple sugars tested showed any capacity to inhibit these agglutinins, and the activity observed was slight in comparison with the inhibition given by the purified A substance.

Detailed investigation of a large number of seed extracts by Renkonen and subsequent workers has revealed that agglutinins reacting preferentially with O or A cells are encountered much more frequently than are those reactive with B cells. An agglutinin reactive with B cells to a fairly high titre was reported by Krüpe and Braun (1952) in an extract of the seeds of *Sophora japonica*; the extract, however, also contained agglutinins reactive with group A cells. Absorption experiments carried out by us failed to separate the two agglutinins, and indeed appeared to indicate the presence in the seed extract of an antibody which detected a receptor common to A and B cells. Inhibition tests with purified A

and B substances supported this idea, since the agglutination of A_1, A_2 or B cells by the *Sophora* extract could be inhibited in each instance by either A or B substances. Of the simple sugars tested N-acetylchondrosamine gave the greatest inhibition of agglutination of A_1, A_2 or B cells. This sugar, however, showed only a small fraction of the activity of group A or B substances when the results were expressed in terms either of maximum dilution giving inhibition or molar concentration. With the exception of glucosamine the other sugars showing slight inhibition, *i.e.*, D-chondrosamine, D-galactose, D-fucose and L-arabinose, can all be related structurally to N-acetylchondrosamine and differ only in the nature of the substituents at carbon atoms 2 and/or 6. On the other hand, since D-galactose is a constituent of the A and B substances, it is possible that the capacity of this sugar to inhibit is not related to its structural resemblance to N-acetylchondrosamine. This suggestion is supported by the observation that D-chondrosamine, which is more closely related to N-acetylchondrosamine than is D-galactose, nevertheless inhibits less well than the latter sugar. It is of interest in this respect to note that lactose, a β-linked galactoside, is slightly more active in inhibition tests than is the parent sugar or the α-linked galactoside, melibiose.

One practical point which arises from this work is based on the observation that the agglutination of A_1 cells by an extract of *Vicia cracca* can be inhibited by N-acetylchondrosamine to a dilution of 1 : 3200, whereas N-acetylglucosamine fails to inhibit at a dilution of 1 : 50. This reaction could therefore be used as a serological test for distinguishing between the two acetylhexosamines and for detecting N-acetylchondrosamine in a mixture of hydrolysis products, such as those arising from the mild acid or enzyme decomposition of mucopolysaccharides and mucoids. The inhibition by this same sugar of the agglutination of A_1, A_2 or B cells by the *Sophora japonica* extract and the inhibition by N-acetylglucosamine of the agglutination of O cells by *Lotus* agglutinin could be used to provide confirmatory evidence for the presence or absence of the two acetylhexosamines. The results of the inhibition of these reagents are less useful than are those obtained with the *Vicia* agglutinin, because the *Lotus* and *Sophora* extracts are neutralised by other sugars which are likely to accompany the acetylhexosamines in the hydrolysis products of polysaccharides.

It is of interest that out of all the agglutination systems examined for inhibition with simple sugars (Watkins and Morgan, 1952, and present observations), the two agglutinins which are inhibited by L-fucose, *Lotus tetragonolobus* extract and eel serum, are both preferentially reactive with O cells, whereas the seed extracts which are inhibited by N-acetylchondrosamine have anti-A (*Vicia cracca* and Lima bean) or anti-A and anti-B (*Sophora japonica*) specificity. Other evidence in support of the greater importance of L-fucose for H specificity is indicated from the results of chemical investigations (Annison and Morgan, 1952*b*, and unpublished observations.) The significance of the inhibition reactions obtained with simple sugars in relation to the specificity of the A, B and H substances, however, cannot be estimated fully until our knowledge of the detailed chemical structure of these substances is more complete.

SUMMARY.

Aqueous extracts of seeds from *Lotus tetragonolobus, Laburnum alpinum, Cytisus sessilifolius, Sophora japonica, Vicia cracca* and Lima bean were examined

for their reactivity with human erythrocytes of different phenotype and for inhibition with blood group substances and simple substances of known structure.

The agglutinins present in *Lotus tetragonolobus*, *Laburnum alpinum* and *Cytisus sessilifolius* reacted preferentially with cells of group O and the agglutination of these cells was inhibited by human and hog H-substances. α-Methyl-L-fucopyranoside inhibited the *Lotus* agglutinin to a higher titre than the H-substance, and inhibition was also obtained with other sugars which differ from L-fucose only by the nature or configuration of the substituents at one or two carbon atoms. The *Laburnum alpinum* and *Cytisus sessilifolius* agglutinins were not neutralised by any of the simple substances tested.

The anti-A agglutinins extracted from *Vicia cracca* and Lima bean seeds were inhibited by human A-substance and weakly by N-acetylchondrosamine.

The agglutination of A_1, A_2 or B cells by the *Sophora japonica* extract was inhibited by either human A or B substance, and absorption experiments indicated that the agglutinin reacted with a structure common to the A and B substances. Slight inhibition was obtained with N-acetylchondrosamine, and to a lesser degree with sugars possessing related structures.

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REFERENCES.

AMINOFF, D., MORGAN, W. T. J., AND WATKINS, WINIFRED M.—(1950) *Biochem. J.*, **46**, 426.

ANNISON, E. F., AND MORGAN, W. T. J.—(1950) *Nature, Lond.*, **165**, 884.—(1952*a*) *Biochem J.*, **50**, 460.—(1952*b*) *Ibid.*, **52**, 247.

BIRD, G. W. G.—(1952) *Indian J. med. Research*, **40**, 289.

BOYD, W. C.—(1950) *J. Immunol.*, **65**, 281.

Idem AND REGUERA, R. M.—(1949) *Ibid.*, **62**, 333.

CAZAL, P., AND LALAURIE, M.—(1952) *Acta haemat.*, **8**, 73.

CUSHING, J. E.—(1952) *Science*, **115**, 404.

ELO, J., ESTOLA, E., AND MALMSTRÖM, N.—(1951) *Ann. Med. exp. Fenn.*, **29**, 297.

FEINBERG, J. G., AND MORGAN, W. T. J.—(1953) *Brit. J. exp. Path.*, (in press).

GIBBONS, R. A., AND MORGAN, W. T. J.—(1952) *Nature, Lond.*, **170**, 77.

GRUBB, R.—(1949) *Acta path. microbiol. scand.*, Suppl. 84.

HOOKER, S. B., AND ANDERSON, L. M.—(1921) *J. Immunol.*, **6**, 419.

KEKWICK, R. A.—(1952) *Biochem. J.*, **52**, 259.

KOULUMIES, R.—(1949) *Ann. Med. exp. Fenn.*, **27**, 185.

KRÜPE, M.—(1950) *Z. ImmunForsch.*, **107**, 450.

Idem AND BRAUN, C.—(1952) *Naturwissenschaften*, **39**, 284.

LANDSTEINER, K.—(1947) ' The Specificity of Serological Reactions.' Cambridge, Mass. (Harvard University Press).

MARRACK, J. R., AND CARPENTER, B. R.—(1938) *Brit. J. exp. Path.*, **19**, 53.

MORGAN, W. T. J.—(1937) *Biochem. J.*, **31**, 2003.

Idem AND WADDELL, M. B. R.—(1945) *Brit. J. exp. Path.*, **26**, 387.

RACE, R. R., AND SANGER, R.—(1950) ' Blood Groups in Man.' Oxford (Blackwell).

RENKONEN, K. O.—(1948) *Ann. Med. exp. Fenn.*, **26**, 66.—(1950) *Ibid.*, **28**, 45.

WATKINS, WINIFRED M., AND MORGAN, W. T. J.—(1952) *Nature, Lond.*, **169**, 825.

WIENER, A. S.—(1943) ' Blood Groups and Transfusion.' 3rd ed. Springfield (Thomas).

THE ISOLATION OF A SPECIFIC SUBSTANCE AND A GLYCOGEN-LIKE POLYSACCHARIDE FROM *TRICHOMONAS FOETUS* (VAR. MANLEY).

J. G. FEINBERG AND W. T. J. MORGAN.

From the Lister Institute of Preventive Medicine, London, S.W.1.

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Trichomonas foetus is a protozoan flagellate which inhabits the genital tract of bovines and is responsible for much of the abortion and infertility in this species. In the bull it inhabits the preputial cavity, from which it is transmitted to the cow by coitus and subsequently proliferates in the uterus.

Kerr and Robertson (1945) distinguished two varieties of the organism on the basis of immunological behaviour in bovines and laboratory animals and designated the varieties " Manley " and " Belfast."

Svec (1944) attempted to isolate specific substances from *T. foetus*, using a modification of the method introduced by Fuller (1938) for the isolation of specific carbohydrates from streptococci. The culture was extracted with hot formamide and the soluble material separated into several fractions by the addition of alcohol. The carbohydrate isolated failed to induce a skin reaction in artificially immunized cows, and at high concentration gave only poor precipitation with bovine immune *T. foetus* anti-sera. These fractions and other preparations were examined further by B. B. Morgan (1948) in an unsuccessful attempt to elicit skin reactions in cattle and obtain precipitation reactions with the serum of rabbits and cattle immunized with *T. foetus* cultures. MacDonald and Tatum (1948) also reported they were unable to obtain a specific precipitation with these preparations and the serum of rabbits inoculated either with the supernatant fluid from old cultures of *T. foetus* or with materials obtained by the extraction of organisms with formamide or as a result of sonic disruption.

The present paper describes the isolation of a material from *T. foetus* which possesses the same dominant serological specificity as the intact protozoon. The specific substance is extracted from the organism with anhydrous diethylene glycol, a procedure already extensively developed (Morgan, 1937 ; Henderson and Morgan, 1938 ; Morgan and Partridge, 1940, 1941 ; Partridge and Morgan, 1942 ; Morgan, 1949) for the extraction of specific antigenic materials from certain organisms of the genera *Shigella* and *Salmonella*. The isolation of a serologically inactive polysaccharide, most probably glycogen, from *T. foetus* is also described.

MATERIALS AND METHODS.

Trichomonas foetus.—The organisms were grown in pure culture for 48 hr. at 37° in 1 l. quantities in a glucose serum broth medium (Kerr and Robertson, 1947) to reach a density of about 5×10^6 cells per ml. The organisms were collected and washed with sterile saline by centrifugation and immediately dried from the frozen state. About 0·5 g. dry organisms was obtained from each litre of culture fluid.

Organic solvents.—Commercial ethylene glycol (b.p. 197°) and diethylene glycol (b.p. 245°) were re-distilled and only the constant boiling material was collected for use.

Agglutination tests.—Performed by the method of Robertson (1941) using Petri dishes to observe agglutination. A capillary agglutination test introduced by Feinberg (1952) was also used.

Precipitation tests.—A capillary precipitation test similar to that described by Dubos (1948) for typing streptococci was used with certain modifications.

Nitrogen content.—Determined by the Kjeldahl method using the apparatus of Markham (1942) and a mixed bromocresol green and methyl red indicator (Ma and Zuazaga, 1942).

Phosphorus.—Estimated by the method of Martland and Robison (1926).

Reducing power.—Estimated by the method of Somogyi (1937).

Hexosamine.—Determined by a modification of the method of Elson and Morgan (1933) using glucosamine concentrations of 10–40 μg. as standards, a photoelectric colorimeter and a green light filter (λ, 540 mμ.).

Rhamnose.—Estimated by the method of Dische and Shettles (1948).

Chromatographic methods.—Paper chromatography as developed by Consden, Gordon and Martin (1944), using as solvents collidine, phenol and *n*-butanol and Whatman No. 1 paper, was used for the qualitative examination of the amino-acids, hexosamines and sugars. The amino-acids were detected by spraying the papers with ninhydrin. The sugar spots were developed by spraying with $AgNO_3/NH_3$ and heating to 105° for 5 min. (Partridge, 1946), or by treatment with aniline hydrogen phthalate (Partridge, 1949). The hexosamine was detected on the paper sheets or strips as cherry red areas, by spraying with an alkaline acetylacetone reagent followed by *p*-dimethylaminobenzaldehyde in HCl-*n*-butanol (Partridge, 1948).

Isolation of the Specific Substance.

The freeze-dried organisms were extracted successively with alcohol, ether, acetone, chloroform and pentane and the extracted culture freed from solvent by drying *in vacuo*. The material was then quickly ground with ten times its weight of freshly distilled diethylene glycol, immediately transferred to a glass-stoppered bottle and allowed to stand in the dark at room temperature for several days. Each day the suspension was gently shaken to ensure thorough mixing; vigorous shaking is to be avoided, as such treatment leads to the formation of stable foams which are difficult to handle. The suspension was finally centrifuged in the closed bowl of a Sharples supercentrifuge (25,000 r.p.m.) until it was apparently free from suspended organisms. The diethylene glycol extract was recovered as a clear pale yellow fluid. The organisms were scraped from the bowl and re-suspended in about one-half the original volume of solvent, and the whole process described above repeated. Several extracts were prepared thus. The diethylene glycol extracts were filtered through a Berkfeld candle to remove any intact organisms and cellular debris which remained. In earlier experiments the filtered solutions were dialysed at 1–2° against distilled water until free from solvent, concentrated to a small volume *in vacuo* at 15–20° and the soluble materials recovered by drying from the frozen state. The substances so obtained were sometimes found to contain a small amount of lipoid material which made them difficult to handle. In later experiments, in order to avoid this lipoid contaminant, the filtered diethylene glycol extracts were poured into 4 times their volume of cold acetone, vigorously stirred and allowed to flocculate at 1–2° for 24 hr. The materials thrown out of solution were collected by centrifugation, washed with a 1:4 diethylene glycol-acetone mixture and then with acetone. The deposits were dissolved in water, dialysed to remove acetone and the solutions evaporated to dryness from the frozen state. The amount of material recovered from 79 g. dry *T. foetus* culture by each of 5 successive diethylene glycol extracts is recorded in Table I. The quantity of substance extracted rapidly decreases, and the results indicate that for practical purposes 3 extractions remove the bulk of the material extractable with diethylene glycol.

Material precipitated from an aqueous solution of the diethylene glycol soluble material at 80 v/v per cent acetone concentration showed considerable *T. foetus* specificity, whereas that remaining in the 80 v/v per cent acetone supernatant fluid was serologically inactive. The specific substance (375 mg.) was purified by fractional precipitation from aqueous solution (16 ml.) by acetone. The solution became heavily opalescent on the addition of 11 ml. acetone and a precipitate formed on the addition of a further 1 ml. acetone. The precipitate was collected by centrifugation, washed with acetone and dried. The addition of acetone to the supernatant fluid gave additional material and in all five fractions were

collected by increasing the acetone concentration, but the general behaviour of the solution during fractionation suggested that no sharp differentiation of the materials present was to

TABLE I.—*The Extraction of Dry* T. foetus *Culture* (79 *g.*) *with Anhydrous Diethylene Glycol.*

Extraction number.	Volume of diethylene glycol added (ml.).	Volume of solvent recovered (ml.).	Amount of material in extract (mg.).	Percentage yield*.
1	800	434	435	0·55
2	600	574	275	0·31
3	450	450	140	0·18
4	300	275	40	0·05
5	200	240	34	0·04

* Expressed on the weight of dry *T. foetus* used.

be expected. The material in each fraction was dissolved in water, dialysed and recovered by drying from the frozen state. The amount of material recovered in each fraction is given in Table II. The above experiment was repeated using a further and equal amount of the

TABLE II.—*The Fractionation from Aqueous Solution by Acetone of the Material Extracted from* T. foetus.

Fraction number.	Acetone concentration (v/v per cent).	Weight of material (mg.).	End-point of specific precipitation.*
1	0–43	144	1:32,000
2	43–66	118	1:32,000
3	66–81	14	1:32,000
4	81–84	4	—
5	above 84	—	—

* Tested with the serum of rabbits immunized with living *T. foetus*.

primary diethylene glycol extract. In this instance, however, a single main fraction obtained over the range 40–80 v/v per cent acetone concentration was collected. The material recovered on the addition of acetone up to 40 v/v per cent concentration was equivalent to 5 per cent of the starting material, whereas the main fraction (40–80 v/v per cent acetone) represented rather more than 60 per cent.

A number of similar preparations of material precipitated over the range 40–80 per cent acetone concentration were mixed and the material (1·18 g.) was again fractionated from aqueous solution by the addition of acetone. Fractions separating between 0 and 43 v/v per cent, 43 and 75 v/v per cent and material remaining in solution at 75 v/v per cent acetone were obtained. Details of the experiment and analytical figures for the substances obtained are given in Table III. Fraction 2, Table III, was found to be more active serologically

TABLE III.—*Analytical Figures for the Material Obtained from* T. foetus.

Fraction number.	Acetone concentration (v/v per cent).	Amount of material recovered (mg.).	Percentage of total material recovered.	$(\alpha)_{5461}$.	N per cent.	P per cent.	End-point of specific precipitation.*
1	0–43	148	15	−60°	3·4	1·9	1:16,000
2	43–75	676	68	−62°	3·3	1·8	1:64,000
3	above 75	165	17	−56°	4·4	1·5	1:32,000

* Tested with the serum of rabbits immunized with living *T. foetus*.

than either of the other fractions, and was accordingly accepted as the best preparation of *T. foetus* specific substance and was examined in greater detail.

Properties of the Specific Substance.

Homogeneity.—The material (Table III, fraction 2) was examined in a Tiselius electrophoresis apparatus at 0° using the diagonal schlieren optical system and monochromatic light, $\lambda = 546$ mμ. In acetate buffer pH 4, $I = 0{\cdot}1$, and in phosphate buffer pH 8, $I = 0{\cdot}2$; after prolonged electrophoresis only one component was demonstrable (Fig. 1). An ultracentrifugal examination was kindly

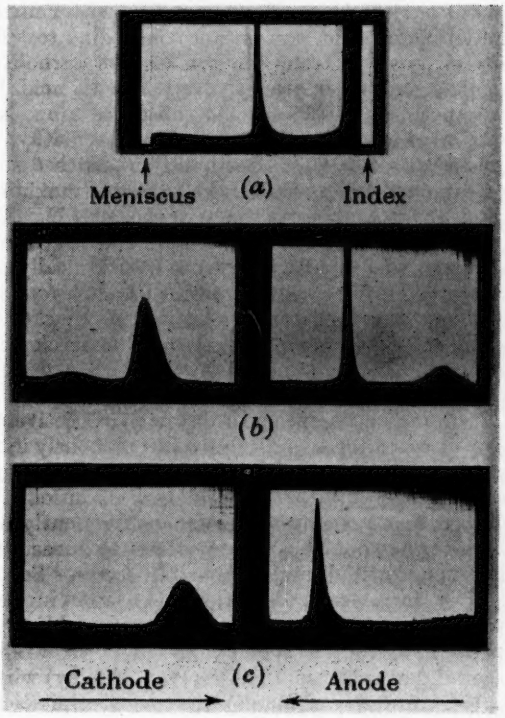

FIG. 1.—Ultracentrifuge and electrophoresis diagrams of *T. foetus* specific substance. (*a*) Ultracentrifuge diagram of a 1 w/v per cent solution in phosphate buffer (pH 8·0, $I = 0{\cdot}2$) after 50 min. at 270,000 g. (*b*) Electrophoresis diagram of 1·5 w/v per cent in phosphate buffer (pH 8·0, $I = 0{\cdot}2$) after 150 min. at 5 V./cm. (*c*) Electrophoresis diagram of 1·1 w/v per cent solution in acetate buffer (pH 4·0, $I = 0{\cdot}2$) after 190 min. at 5 V./cm. Migration direction in (*b*) and (*c*) shown by arrows.

carried out by Mr. H. Murray using a Svedberg oil turbine machine at 60,000 r.p.m. (270,000 g.). Solutions of the material were made up in buffers, and sufficient NaCl was present to supress any charge effect on sedimentation. The absence of spreading of the boundary after prolonged centrifugation suggests a high degree of homogeneity of particle size.

Ultraviolet absorption.—The specific substance (1 v/v per cent in water) was examined in a "Uvispek" spectrophotometer. No significant amount of absorption was shown between 260 mμ. and 310 mμ.

Optical rotation.—The specific rotation, the mean value from the examination of several preparations is $(\alpha)_{5461} - 62°$ in water (c, 1·0).

Chemical properties.—The specific substance dissolves readily in water and a 2 per cent solution gives no precipitate on the addition of an equal volume of 10 per cent trichloroacetic acid, salicylsulphonic acid (20 per cent), tannic acid (10 per cent), saturated picric acid, mercuric acetate or silver nitrate (2 per cent). The biuret and ninhydrin tests for protein and the iodine test for glycogen are negative. The material gives a strong Molisch test for carbohydrate, but does not reduce Fehling's reagent until after hydrolysis with acid. The analytical figures for a typical specimen of the specific substance are: N, 3·2 per cent; P, 1·8 per cent, none of which is free inorganic P. The methyl pentose content is 30 per cent, expressed as L-fucose. After hydrolysis with 5 N HCl for 8 hr. at 100° the values for α-amino N and α-amino-acid N reach a maximum, representing about 75 per cent and 37 per cent respectively of the total N. These determinations were kindly made by Mr. R. A. Gibbons.

The specific substance was rapidly inactivated serologically, as measured by the failure of the material to form a precipitate with a *T. foetus* immune rabbit serum, by a purified enzyme preparation obtained from a *Cl. welchii* culture filtrate (Stack and Morgan, 1949) which destroys the serological properties of the A, B and H human blood group mucoids.

Immunological properties.—Antisera containing specific agglutinins and precipitins for living *T. foetus* or its specific substance respectively were prepared by injecting living or dried cultures of *T. foetus* intravenously in rabbits.

Rabbits given 300 mg. (dry wt.) of living culture or dried organisms divided into 5 doses generally respond with the formation of specific agglutinins and precipitins, and quite potent precipitating sera are frequently obtained. These sera have been used throughout the present work to measure the serological activity of purified materials obtained from *T. foetus*. Several intradermal (0·15–0·30 mg.) and/or intravenous injections (5·0–12·0 mg.) of the purified diethylene glycol extract failed to induce any demonstrable specific agglutinin and precipitin response in a group of rabbits.

The soluble material derived from *T. foetus* (var. Manley) when tested against undiluted anti-sera to *T. foetus* (var. Manley) gave a precipitation end-point at a dilution of 1:64,000, whereas tested against an antiserum induced by immunization with *T. foetus* (var. Belfast) or against normal rabbit serum the end-titre was never more than 1:1000 and usually less than 1:200.

Kerr (1944) demonstrated that an immediate (allergic type) skin reaction can be elicited in cattle infected or immunized with *T. foetus* by a trichloroacetic acid extract of the organism ("Tricin"). It was thus of interest to determine if the purified specific hapten obtained in the present work could replace "Tricin" as a diagnostic reagent for field trials. Our results do not provide a direct answer to this question, but the following observations indicate that the development of such a reagent may be anticipated. The only cattle available to us for experimental purposes were artificially infected or immunized with *T. foetus* (var. Belfast), and were therefore unsuitable as test animals for the assay of the specific substance derived from the Manley strain. A preparation of hapten was, how-

ever, made by us from *T. foetus* (var. Belfast) and kindly tested by Dr. Kerr, who has allowed us to quote some of the results from his protocols (Table IV).

TABLE IV.—*Skin Reactions in Cows Sensitized to* T. foetus *(var. Belfast).*

Site.	Conc. test solution (mg./ml.).	Increase in skin thickness (mm.).							
		Calf E.		Heifer K 10.		Cow D 13.		Normal cow.	
		0·5 hr.	1 hr.	0·5 hr.	1 hr.	0·5 hr.	1 hr.	0·5 hr.	1 hr.
1	1·0	8·5	7·5	6·0	6·0	7·0	7·0	1·0	1·0
2	0·5	6·5	6·0	5·0	5·0	6·5	5·5	0	0
3	0·25	4·0	5·0	4·0	5·0	3·0	4·0	0	0
4	0·125	5·0	5·5	1·0	1·5	3·0	3·5	0	0

Intradermal injections (0·1 ml.) were made on the sides of the neck, the sites being numbered in clockwise direction on the left side of the neck and counter-clockwise on the right side, so that all sites with similar numbers are strictly comparable in anatomical location. The readings given in Table IV represent the increase in thickness of the double layer of skin in a fold at the site of injection, *i.e.*, the total thickness of the injected skin less the initial thickness measured with micrometer calipers before injection. The reaction is prompt, becoming evident within 10 min., reaching its peak in about 30 min.–1 hr., and thereafter fading rapidly until it has almost disappeared after 6 hr. An increase in thickness of 3 mm. or more is considered significant. The results obtained with three sensitized animals (Calf 7, Heifer K 10, and Cow D 13) and one normal cow are given in Table IV. The soluble specific substance derived from *T. foetus* (var. Belfast) produces a local swelling when injected into the skin of cattle sensitized to this organism whereas the same material injected into similar sites in a normal animal elicits no reaction. The animals were not tested with the specific soluble substance derived from the Manley variety of *T. foetus* or with other similar but unrelated polysaccharide materials ; it is therefore not possible to be certain that the reaction observed with the extract of the homologous organism is entirely specific. Further intradermal tests with the Belfast hapten (1 mg./ml.) were subsequently made by Dr. Kerr on 5 unselected bovines presented for slaughter at the Belfast abattoir. Four of the animals gave little or no reaction (0·5 to 1·0 mm.), while in the fifth, a cow, a low positive reading of 4·5 mm. was obtained.

Skin tests were also carried out on rabbits which had been given a series of intraperitoneal injections of freeze-dried *T. foetus* totalling about 800 mg. The test substances were (a) saline, (b) the glycogen-like material (see below), (c) the specific substance, and (d) laked whole *T. foetus* organisms. The intradermal injections were made on the shaved backs of the rabbits 7 days after the last intraperitoneal dose had been given, and each test material was injected as 0·1 ml. of a 0·5 w/v per cent solution. Two normal rabbits were tested similarly as controls. The results of the tests are summarized in Table V. Preparations (c) and (d) caused definite skin reactions in the rabbits (Nos. 1, 2 and 3) immunized with whole organisms, whereas materials (a) and (b) failed to induce a similar reaction. The normal animals (Nos. 6 and 7) gave no significant reaction with any of the reagents. The rabbits which reacted with reagents (c) and (d) gave a delayed type of skin reaction and in so reacting behaved very differently from

TABLE V.—*Skin Reactions Following the Intradermal Injection of Materials into Rabbits Immunized with* T. foetus *Preparations.*

Time of observation after intradermal inoculation (hr.).	Test substance.	Rabbits immunized with— Whole *T. foetus* culture. 1.	2.	3.	*T. foetus* glycogen. 4.	5.	Uninoculated rabbits. 6.	7.
0·5–1·0	a	0	±	0	±	0	±	0
	b	±	±	±	±	±	±	0
	c	+	+	+	++	+	±	±
	d	++	+	+	++	++	0	0
5	a	0	0	0	0	0	0	0
	b	0	0	0	+	0	0	0
	c	+++	++	+	+±	+	±	±
	d	+++	+++	+	+±	+	±	±
24	a	0	0	0	0	0	0	0
	b	0	0	0	0	0	0	0
	c	+++	++	++	0	±	±	±
	d	+++	++	++	0	±	±	±

a, saline. b, *T. foetus* glycogen. c, *T. foetus* specific substance. d, laked *T. foetus* culture.
0, no reaction. ±, very slight reaction. +, definite reaction. ++, strong reaction.
+++, very strong reaction.

cattle, where an immediate reaction ensued. Twenty-four hours after the intradermal injection of (c) and (d) the reaction site showed a large diffuse swelling with some induration and erythema.

Acid Hydrolysis of the Specific Substance.

The substance was hydrolysed with 0·5 N and 6 N HCl in small sealed glass ampoules heated for the appropriate time in a boiling water-bath. The solutions were exactly neutralised with 0·5 N NaOH, or in the case of the stronger acid, known amounts were evaporated to dryness *in vacuo* in small glass dishes over pellets of NaOH and concentrated H_2SO_4. The rate of liberation of reducing substances and of hexosamine during hydrolysis with 0·5 N HCl is given in Fig. 2 and 3. The results indicate that the maximum liberation of reducing sugars, 46 per cent (expressed as glucose), and of hexosamine, about 9 per cent (expressed as glucosamine base), occur at approximately the same time (8–16 hr.). After heating for this time with 1 v/v per cent acetic acid the material gives about 6 per cent reduction in terms of glucose (Fig. 2), and less than 5 per cent of the total phosphorus is liberated as inorganic phosphate. The specific substance, after heating for a short time with 0·1 N HCl, gives rise to a white precipitate which dissolves readily in dilute alkali and is thrown out of solution at pH values below 7. The substance was found to contain nitrogen and organic phosphorus but has not been further investigated. The rate of liberation of phosphorus from the specific substance during hydrolysis with 2 N HCl at 100° was followed over a period of 72 hr. The results (Fig. 4) show that about 90 per cent of the total phosphorus was liberated as inorganic phosphate. Under the same conditions of hydrolysis sodium β-glycerophosphate liberated 60 per cent of its total phosphorus as inorganic phosphate. The products of complete hydrolysis with 6 N HCl for

16 hr. at 100° were examined by partition chromatography using 1 mg. of the material. The chromatogram was run in two directions at right angles with collidine (48 hr.) and phenol (20 hr.). Treatment of the chromatogram with

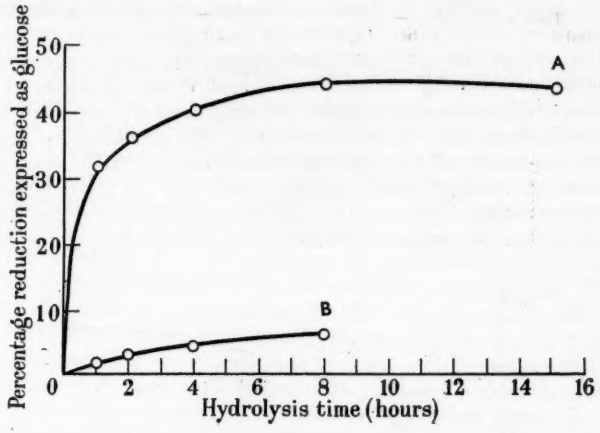

FIG. 2.—Hydrolysis of the specific substance (A) by 0·5 N HCl at 100°, (B) by N-acetic acid at 100° as determined by reducing power, expressed as glucose.

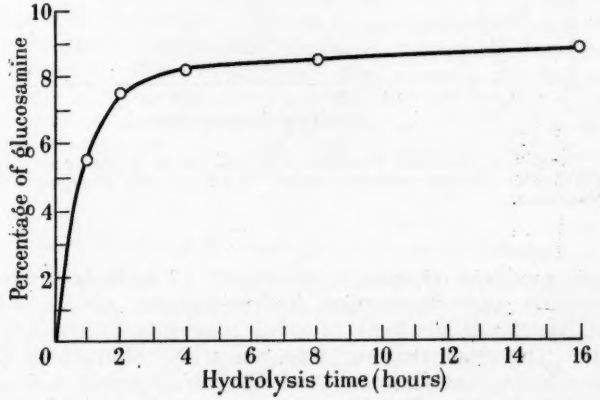

FIG. 3.—The liberation of glucosamine from the specific substance during hydrolysis with 0·5 N HCl at 100°.

ninhydrin containing 1 per cent acetic acid according to the technique of Consden and Gordon (1948) and subsequent heating at 80–90° for 5 min. developed eleven mauve spots. The chromatogram was compared with that obtained with an artificial mixture of known amino-acids run at the same time. Ten of the spots

given by the specific substance corresponded closely in position with those which were given by the following amino-acids : lysine, arginine, aspartic acid, glutamic acid, glycine, serine, alanine, threonine, valine and leucine (*iso*leucine).

The hexosamines, glucosamine and chondrosamine, give under these conditions greyish-mauve spots similar to those developed by amino-acids. The amino-sugars are, however, readily distinguishable from amino-acids on the chromatogram with the aid of the *p*-dimethylaminobenzaldehyde reagent described by Partridge (1948), and by the use of this reagent it was possible to identify the eleventh spot on the two-dimensional chromatogram described above as an amino-sugar. Confirmation of this conclusion was obtained by examination of the hydrolysis products of the specific substance on strip chromatograms as described earlier by Aminoff and Morgan (1948). Along the width of the paper six positions were marked out and the following substances (or mixtures) put on the paper : (*a*) chondrosamine hydrochloride, (*b*) chondrosamine hydrochloride

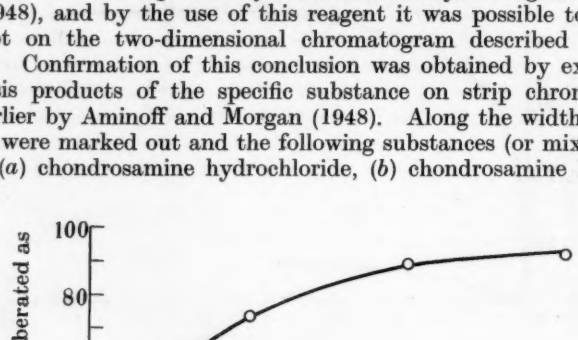

FIG. 4.—The formation of inorganic phosphate from the specific substance during hydrolysis with 2 N HCl at 100°. Results expressed as per cent of the total phosphorus liberated as inorganic phosphate.

and hydrolysis products of specific substance, (*c*) hydrolysis products alone, (*d*) chondrosamine and glucosamine hydrochlorides, (*e*) glucosamine hydrochloride and hydrolysis products of specific substance, and (*f*) glucosamine hydrochloride. The chromatogram was run with collidine for 72 hr., dried, sprayed with alkaline acetylacetone and Ehrlich's reagent and heated to 90° for 5 min. as described by Partridge (1948). A single reddish spot developed in similar areas below positions (*c*), (*e*) and (*f*), whereas two similar spots each appeared on strips (*b*) and (*d*). There is evidence, therefore, that glucosamine is the only hexosamine present in the specific substance.

One-dimensional paper chromatograms were run with collidine-water phenol-NH_3 or *n*-butanol (40 per cent), acetic acid (10 per cent) and water (50 per cent) as solvent systems, and the distribution and number and position of sugars present determined by means of $AgNO_3/NH_3$ or aniline hydrogen phthalate

reagents. Rhamnose, fucose, galactose and xylose were identified as component sugars. A weak and somewhat doubtful spot indicating the possible presence of glucose was also detected. However, owing to the presence of considerable amounts of a polyglucose in the organism, it would be unwise to accept glucose as a component of the specific substance without additional evidence.

The Isolation of a Glycogen-like Material from T. foetus.

It was observed that a material which gave an intense Molisch reaction and a typical brownish-red coloration with iodine solution was present in aqueous extracts of *T. foetus* and in thick suspensions of the organisms which had been disintegrated by ultrasonic waves. The substance possessed none of the specific serological character associated with *T. foetus*, and it seemed probable that the material was largely a storage carbohydrate similar to glycogen. In order to investigate the substance further cultures of *T. foetus* which had been exhaustively treated with anhydrous diethylene glycol and had yielded all the *T. foetus* specific substance which could be extracted under the experimental conditions with this solvent were further extracted with anhydrous ethylene glycol. By this means a carbohydrate material equivalent to between 5 and 10 per cent of the weight of the dry culture employed was selectively removed from the organism. The substance was readily recovered from the organic solvent, after it had been centrifuged at high speed and passed through a Berkfeld filter to remove any organisms or protozoal debris, by the addition of acetone to yield a concentration of 33 v/v per cent. The precipitate was re-dissolved in water and re-precipitated twice from aqueous solution with half its volume of acetone. The material gave a strongly opalescent aqueous solution which was cooled to 0°, and 4 w/v per cent trichloroacetic acid was added. The small precipitate which formed was discarded, and the main carbohydrate component recovered from solution by the addition of an equal volume of acetone. The material was dissolved in water, glacial acetic acid added to yield a concentration of 80 v/v per cent, the precipitate formed recovered by centrifugation, dissolved in water and re-precipitated at the same concentration of acetic acid. The substance, which gave a colourless opalescent aqueous solution, was thoroughly dialysed and dried from the frozen state.

The Properties of the Serologically Inactive Carbohydrate.

A 2 w/v per cent aqueous solution of the material gives an intense brownish-red coloration on the addition of iodine solution. The coloration disappears on heating the solution and reappears on cooling. A strong Molisch reaction is given by the solution, which, however, fails to reduce Fehling's solution until after treatment with hot mineral acid. The ninhydrin and biuret reactions are negative, and the addition of trichloroacetic acid (10 w/v per cent), salicylsulphonic acid (20 w/v per cent), tannic acid (10 w/v per cent), saturated aqueous picric acid, mercuric acetate and silver nitrate fail to give rise to a precipitate. The material is, however, precipitated by basic lead acetate.

The substance shows a strong dextro-rotation, $(\alpha)_{5461} + 196°$ (c, 1 per cent), and gives no pronounced or characteristic absorption bands when examined in a "Uvispek" spectrophotometer over the wave-length range 220–310 mμ. The material contains neither nitrogen nor phosphorus, and gives rise to a maximum

of about 105 per cent reduction, in terms of glucose, after hydrolysis for 8 hr. with 0·5 N HCl at 100°. The rate of hydrolysis was found to be identical with that shown by a purified preparation of liver glycogen hydrolysed under the same conditions. The hydrolysis products gave glucosazone (m.pt. 198°) when treated with phenylhydrazine. Mixed m.p. with an authentic specimen of glucosazone, 197°.

The products of hydrolysis of the carbohydrate material with 0·5 N HCl at 100° were examined on strip chromatograms using phenol or collidine as the organic phase. Glucose and acid hydrolysis products of glycogen were run on the chromatogram as control substances. The two polysaccharide materials gave identical chromatograms when the products arising from each after 30 min., 4, 16, and 24 hr. hydrolysis were compared. After complete hydrolysis of the serologically inactive carbohydrate, glucose was the only sugar demonstrable on the chromatogram. The carbohydrate material derived from *T. foetus* appears to be a polyglucose, indistinguishable from glycogen by the tests carried out.

Immunological Observations.

In view of the observations of Campbell (1936, 1937, 1939, 1942) on the antigenic nature of a series of nitrogen-free polysaccharides which he obtained from certain metazoon organisms, such as *Ascaris lumbricoides*, *Trichinella spiralis* and *Cysticercus crassicollis*, it seemed expedient to examine the immunological behaviour of the glycogen obtained from *T. foetus*. Two rabbits (Table V, No. 4 and 5) were inoculated intraperitoneally at 2–3-day intervals with 15 mg. of the material. Two series of inoculations were given, each of 5 doses and separated by an interval of 5 days. In the last series 20 mg. of the material was given at each injection. Three rabbits (Table V, No. 1, 2 and 3) were similarly immunized each with 800 mg. of freeze-dried *T. foetus* culture. The individual doses varied from 100 mg. to 300 mg. of the dry culture. Two normal rabbits served as controls. Seven days after the last dose each animal was bled and skin-tested. The two rabbits which had received the glycogen-like material and the two normal rabbits showed no significant skin reaction when given the glycogen-like material intradermally (0·1 ml. of 0·5 w/v per cent solution in saline). The two animals which had been immunized with the glycogen-like material also received intradermally the freeze-dried *T. foetus* culture and the specific substance, and gave a definite and immediate reaction which lasted for at least 5 hr. and then disappeared completely. The three rabbits which had been immunized with the whole culture of *T. foetus* gave no skin reaction on receiving the glycogen-like material intradermally. The results of these tests are given in Table V.

Precipitation reaction.—In no instance did the sera of the rabbits which had received intraperitoneal doses of either the whole culture of *T. foetus* or the purified protozoal glycogen give a precipitate on addition of the homologous glycogen preparation.

DISCUSSION.

A method for the isolation of an immunologically specific substance and a glycogen-like carbohydrate from the protozoon, *T. foetus*, is given which is based on the differential extraction of the two materials by means of anhydrous organic

solvents. The possibility of elaborating such a method for the isolation of individual components from the bacterial cell was discussed earlier (Morgan, 1937; Morgan and Partridge, 1941), and such a procedure involving the successive extraction of a thoroughly washed and freeze-dried culture of *T. foetus* with diethylene glycol and ethylene glycol is now described. Anhydrous diethylene glycol removes, at normal temperatures and a neutral pH, not more than a few per cent of the protozoal substance, and the extract contains a substance which possesses a serological specificity very similar to, or identical with, that of the dominant specificity of the living organism. This material, after fractionation from aqueous solution with acetone to remove small amounts of unspecific contaminants, is identified as a polysaccharide-amino-acid complex which precipitates at low concentrations with a *T. foetus* immune rabbit serum but fails to engender specific agglutinins or precipitins in rabbits under the conditions of immunization employed. These results suggest that the specific substance obtained is probably not the complete antigen as it exists in the living or freeze-dried culture, but that it is an important constituent of the antigen and functions as the specific determinant, and might be classified provisionally as a hapten. The absence of antigenicity when the material is tested in rabbits is similar to that recorded for preparations of the human blood-group substances, which are likewise complexes of polysaccharide and amino-acid-containing residues. The blood-group substances are, however, powerful antigens when tested in man (see Witebsky, Klendshoj and McNeil, 1944; Wiener, Soble and Polivka, 1945), and it may well be that in a different animal species the specific material obtained from *T. foetus* would similarly prove capable of inducing the formation of specific antibodies.

Although only preliminary observations on the chemistry of the specific substance have yet been made, it is already evident that this immunologically important material consists of a complex carbohydrate to which are firmly bound amino-acids, most probably in the form of a complex amino-acid-containing residue. Quantitatively, the main sugar component (30 per cent) is methylpentose, which is, however, not composed of a single sugar but is a mixture of fucose and rhamnose. Chromatographic analysis reveals the presence of galactose and xylose and the amino-sugar glucosamine as well as the two methylpentoses. The low content of glucosamine (9 per cent) in the specific substance suggests that most of the nitrogen (3·2 per cent) in the material arises from the amino-acids present, which are, incidentally, found to be similar qualitatively to those present in the human specific blood-group substances. Aromatic and sulphur-containing amino-acids appear to be absent. The organic phosphorus is converted slowly to inorganic phosphate during hydrolysis of the specific substance with 2 N HCl at 100°, but there still remains after 72 hours about 10 per cent of the total phosphorus in organic combination. There is no evidence that the hexosamine liberated during hydrolysis fails to reach its maximum value before the whole of the phosphorus is liberated as inorganic phosphorus, as has been reported by Bendich and Chargaff (1946) for the O antigen of Proteus OX 19.

Compared with many bacteria *T. foetus* in the living or in the dried state is a poor antigen. However, antibodies specific for *T. foetus* are readily produced by injecting whole cultures or freeze-dried preparations into rabbits. The specific soluble substance isolated from *T. foetus* (var. Manley), the strain commonly met with in England, when tested with an antiserum to *T. foetus* (var.

Belfast), a strain predominant in Northern Ireland, shows only a weak cross-reactivity, and indicates that the specific substance of the variety Manley is serologically distinct from that of the Belfast variant.

The problems of immunity, sensitization and desensitization in bovines infected or immunized with *T. foetus* have been studied in considerable detail in recent years (Kerr and Robertson, 1941, 1943, 1945, 1946, 1947; Kerr, 1944; Kerr, McGirr and Robertson, 1949), and it has been established in these studies that the value of the skin test in carefully controlled animal experiments is very considerable. Until recently a skin-testing reagent was made from *T. foetus* cultures by laking them with water and precipitating a material from the supernatant fluid with trichloroacetic acid, and the use of the specific material prepared by the extraction of *T. foetus* with ethylene glycol for skin testing has already been favourably commented on by Kerr, McGirr and Robertson (1949), Kerr, Robertson and McGirr (1951), who state that the substance causes no desensitization even when the tests are repeated daily. The results of the skin-testing experiments described in the present paper, using the purified diethylene glycol extract of *T. foetus*, although in many respects inadequate, support these authors' conclusion that the purified specific substance is a useful reagent for the detection of the sensitized state in immunized animals. The absolute specificity of the preparation of the specific substance is not yet completely established, but it is to be hoped that further field experiments may be undertaken.

After repeated extraction of the freeze-dried *T. foetus* culture with anhydrous diethylene glycol no further material passes into solution. If the solvent is now changed and anhydrous ethylene glycol is used a different cellular component, which amounts finally to rather less than 10 per cent of the dry weight of the *T. foetus* culture used, is readily extracted. After several extractions ethylene glycol also fails to remove further material, and to obtain additional constituents from the protozoal bodies it is necessary to employ yet another solvent system. Ethylene glycol, however, is less specific as an extracting agent than is diethylene glycol, and some nucleic acid and nucleoprotein is obtained along with the glycogen-like polysaccharide. Preliminary studies have shown that the nucleoprotein substance possesses *T. foetus* specificity, and it is probably this material which is responsible for reactivity of the " Tricin " and ethylene glycol extracts used by Kerr, McGirr and Robertson (1949, 1951). These materials are separated by repeated precipitation of the polysaccharide from aqueous solution by 33 v/v per cent acetone.

The polysaccharide material in the ethylene glycol extract, after purification, is found to be free from nitrogen, strongly dextro-rotary, and yields glucose only after hydrolysis with mineral acid. This polysaccharide, which possesses no *T. foetus* specific serological character, is most probably a reserve carbohydrate, and is similar to, if not identical with, glycogen. The material fails to induce in the rabbit the formation of anti-sera which precipitate specifically with glycogen, and therefore it behaves in this respect as does mammalian liver glycogen, and is not similar in behaviour to the glycogen isolated from the nematode, *Ascaris lumbricoides*, which according to Campbell (1936) can produce precipitins specific for glycogen in the rabbit.

The *T. foetus* cells after exhaustive extraction with diethylene glycol and ethylene glycol still induce in the rabbit the formation of precipitins and agglutinins specific for *T. foetus*. This observation suggests that the treated cells still

retain a part of the specific substance associated with a cellular component which endows it with full antigenic properties.

SUMMARY.

A method is described for the isolation and purification of a serologically specific substance from the protozoon, *Trichomonas foetus* (var. Manley).

The specific substance, which contains 3·2 per cent N and 1·8 per cent P, is composed of a polysaccharide and an amino-acid-containing moiety, and is strongly laevo-rotatory, $(\alpha)_{5461} - 62°$. It fails to induce the formation of specific agglutinins or precipitins in the rabbit and shows only slight cross-reactivity with *T. foetus* (var. Belfast).

A preliminary examination of the polysaccharide component shows it to contain rhamnose, fucose, xylose, galactose and glucosamine. The amino-acid-containing moiety gives rise to ten amino-acids on hydrolysis.

A polyglucose, most probably glycogen, is identified as a cellular component of *T. foetus*.

The immunological behaviour of the materials isolated is discussed.

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REFERENCES.

Aminoff, D., and Morgan, W. T. J.—(1948) *Nature, Lond.*, **162**, 579.
Bendich, A., and Chargaff, E.—(1946) *J. biol. Chem.*, **166**, 283.
Campbell, D. H.—(1936) *J. infect. Dis.*, **59**, 266.—(1937) *Proc. Soc. exp. Biol., N.Y.*, **36**, 511.—(1939) *J. infect. Dis.*, **65**, 12.—(1942) *Science*, **96**, 431.
Consden, R., and Gordon, A. H.—(1948) *Nature, Lond.*, **164**, 443.
Idem and Martin, A. J. P.—(1944) *Biochem. J.*, **38**, 224.
Dische, Z., and Shettles, L. B.—(1948) *J. biol. Chem.*, **175**, 595.
Dubos, R. J.—(1948) ' Bacterial and Mycotic Infections of Man.' Philadelphia (Lippincott).
Elson, L. A., and Morgan, W. T. J.—(1933) *Biochem. J.*, **29**, 1824.
Feinberg, J. G.—(1952) *J. Path. Bact.*, **64**, 645.
Fuller, A.—(1938) *Brit. J. exp. Path.*, **19**, 130.
Henderson, D. W., and Morgan, W. T. J.—(1938) *Ibid.*, **19**, 82.
Kerr, W. R.—(1944) *Vet. Rec.*, **56**, 303.
Idem and Robertson, M.—(1941) *Vet. J.*, **97**, 351.—(1943) *J. comp. Path.*, **53**, 280.—(1945) *Vet. Rec.*, **57**, 221.—(1946) *J. comp. Path.*, **56**, 38, 101.—(1947) *Ibid.*, **57**, 301.
Idem, McGirr, J. L., and Robertson, M.—(1949) *Ibid.*, **59**, 133.
Idem, Robertson, M., and McGirr, J. L.—(1951) *J. Hyg., Camb.*, **49**, 67.
Ma, T. S., and Zuazaga, G.—(1942) *Industr. Engng. Chem. (Anal.)*, **14**, 280.
MacDonald, E. M., and Tatum, A. L.—(1948) *J. Immunol.*, **59**, 309.

MARKHAM, R.—(1942) *Biochem. J.*, **36**, 790.
MARTLAND, M., AND ROBISON, R.—(1926) *Ibid.*, **20**, 847.
MORGAN, B. B.—(1948) *J. cell. comp. Physiol.*, **32**, 235.
MORGAN, W. T. J.—(1937) *Biochem. J.*, **31**, 2003.—(1949) 'The Nature of the Bacterial Surface.' Oxford (Blackwell).
Idem AND PARTRIDGE, S. M.—(1940) *Biochem. J.*, **34**, 169.—(1941) *Ibid.*, **35**, 1140.
PARTRIDGE, S. M.—(1946) *Nature, Lond.*, **158**, 270.—(1948) *Biochem. J.*, **42**, 238.—(1949) *Nature, Lond.*, **164**, 443.
Idem AND MORGAN, W. T. J.—(1942) *Brit. J. exp. Path.*, **23**, 84.
ROBERTSON, M.—(1941) *J. Path. Bact.*, **53**, 391.
STACK, M. V., AND MORGAN, W. T. J.—(1949) *Brit. J. exp. Path.*, **30**, 470.
SVEC, M.—(1944) *J. Bact.*, **47**, 505.
SOMOGYI, M.—(1937) *J. biol. Chem.*, **117**, 771.
WIENER, A. S., SOBLE, R., AND POLIVKA, H.—(1945) *Proc. Soc. exp. Biol., N.Y.*, **58**, 310.
WITEBSKY, E., KLENDSHOJ, N. C., AND MCNEIL, C.—(1944) *Ibid.*, **55**, 167.

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